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Image Analysis of the Duodenal Endocrine Cells in Mice with Particular Regard to Optical Densitometry

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ABSTRACT

The endocrine cells in the murine proximal duodenum have been investigated by means of immunohistochemistry and computerized image analysis. Five endocrine cell types were identified, namely secretin-, gastric inhibitory polypeptide (GIP)-, gastrin-CCK-, somatostatin- and serotonin immunoreactive cells. The number of endocrine cells/mm^3 epithelial cells was estimated and the cell secretory index (CSI) for different endocrine cell types was determined. Furthermore, the optical density of the cellular immunoreactivity and the immunoreactive area in the cell were determined and an index, cell immunoreactivity content was estimated as the optical density multiplied by the immunoreactive area. It has been suggested that the use of this index might better reflect the cellular peptide/amine content than does the CSI. Serotonin-immunoreactive cells were the predominant endocrine cell type, followed by gastrin/CCK-immunoreactive cells. The numbers of secretin-, GIP- and somatostatin immunoreactive cells were almost identical. All endocrine cell types were present both in crypts and in villi, but were, more numerous in the crypts, except for secretin which was more frequent in the villi.

INTRODUCTION

Image analysis first appeared as a readily available technique in 1963 with the introduction of the Quantitative Television Microscope (QTM). QTM was designed by Metals Research Ltd and was intended for use in metallographical

laboratories. An early application in the biological field was the measuring of the size of air spaces in the damaged lung and counting of silver grains in autometallograph (7, 9,11,12). The rapid developments in computer hardware and software made computer image analysis an easy, rapid, precise and objective tool for determining the number and volume of biological structures (3-5, 8, 10). In computerized image analysis the image from video-cameras is digitized into picture elements (pixels). The grey level (i.e. the brightness) of all the pixels in the image can be measured in terms of light transmitted or reflected, or as an optical density value. In coloured images, measurements are taken on the individual colour components within the image. Thus, computerized image analysis, in addition to traditional morphometry, makes it possible to measure the greyness or colour levels of a stained biological structure.

The mouse is an excellent experimental animal. The endocrine cells in the gut of this animal have not previously been subjected to detailed investigation. Apart from somatostatin (6) and peptide YY (PYY) (1) in the small intestine, no other endocrine cell types have been examined in the gut of normal mouse.

The aim of the present study was to quantify duodenal endocrine cells in mice and to use the opportunity offered by computerized image analysis, using optical densitometry to determine an index reflecting the cellular peptide/amine content.

Material and Methods

Animals

Ten mice (5 females and 5 males) aged 3 months and with an average body weight of 30 g (Bom: NMRI strain, B/S Bomholtgård Breeding and Research Centre, Denmark) were used. The mice were housed in cages, 5 mice in each cage, in a room with artificial light from 6 am to 6 pm. The animals were fed a standard pellet diet (Astra-Ewos AB, Södertälje, Sweden) and allowed water ad

libitum. They were kept in the laboratory for 2 weeks prior to sacrifice in a CO_2 chamber, after an overnight fast. The investigation was approved by the local committee on animal ethics, Umeå University.

Immunohistochemistry

After sacrificing the animals, the proximal part of the duodenum was removed and fixed overnight in 4% buffered formaldehyde and embedded in paraffin wax. The deparaffinized sections were rehydrated, and immersed in 0.01% hydrogen peroxide in Tris-HCl buffer, pH 7.6 for 10 min in order to inhibit the endogenous peroxidase activity. To occupy the non-specific binding sites the sections were pre-treated with 1% bovine serum albumin for 10 min. The sections were immunostained by the avidin-biotin complex (ABC) method (Dako A/S,Glostrup,Denmark)as described in detail previously (4). Briefly, The sections were incubated for 20 h with one of the primary antisera (for details concerning the antisera used, see Table 1). They were incubated with biotinylated swine anti-rabbit IgG, diluted 1:200 for 1h. The sections were then incubated for 1h with avidin-biotin-peroxidase complex, diluted 1:100. Staining of the sections was performed in 50 ml Tris buffer containing 10 μl of 30% H_2O_2 and 25 mg diaminobenzidine tetrahydrochloride (DAB), for 15 min. The same ABC-kit and biotinylated swine anti-rabbit IgG was used throughout the study. The incubation was performed at 4°C. Equal parts of avidin and biotinylated peroxidase were mixed with the Tris-buffer and allowed to stand for 1 h at 4°C.

Specificity controls were the same as those described earlier (4). Briefly, the primary antibodies were replaced by non-immune rabbit serum, and the primary antisera pre-incubated for 24 h with the corresponding or structurally related peptide (50-75 μg/ml diluted antibody) at 4°C.

Computerized image analysis

The Quantimet 500MC image processing and analysis system (Leica, Cambridge, England) linked to an Olympus microscope type BX50 was used. The software used in this system was the Leica Windows-based image analysis tool kit "QWIN" (version 1.02) and an interactive programming system Quips (version 1.02).

Table 1. Details of the antisera used. All antisera were raised in rabbit. *Specific for CCK/gastrin C-terminus. **Specific for glucagon N-terminus and cross-reacts with both pancreatic and enteroglucagon.

Antibody against	*Source*	*Working dilution*	*Code no*
Porcine secretin	Eurodiagnostica, Malmö, Sweden	1:1000	R-787502
Bovine gastric inhibitory peptide (GIP)	Eurodiagnostica	1:1600	R-786403-B2
Synthetic human gastrin-17 *	Eurodiagnostica	1:10,000	R-783511
Synthetic human somatostatin	Dakopatts, Glostrup, Denmark	1:1600	A566
Porcine pancreatic glucagon**	Eurodiagnostica	1:2500	B-31
Porcine motilin	Eurodiagnostica	1:5000	R-842206
Serotonin	Eurodiagnostica	1: 600	B 45-1

The number of endocrine cells and their secretory index were determined as described in detail previously (2)using an automated standard sequence analysis operation. Measurements were made on 40 randomly chosen fields (20 from the crypts and 20 from the villi) for each peptide and animal. These fields were chosen from three to five sections, at least 50 μm apart. The sections were examined with X20 objective and each field observed in the monitor represented 0.034 mm^2 area of the tissue.

In Quantimet 500MC the optical density is sampled and coded as a grey level value in the range 0 to 255. Grey level measurements can be calibrated to known standards and Quantimet 500MC utilizes a "look-up table" in which each grey level from 0 to 255 is related to its calibrated value. Using QUIPS, an automated standard sequence operation was created to measure the optical density in different endocrine cell types. The microscope illumination intensity was constant. The operating parameters of the CCD-video camera were kept constant during all the measurements performed in this study. Ten nucleated cells were randomly chosen for each endocrine cell type and animal. Quantification was undertaken with a X40 objective and each pixel corresponded to 0.206 μm.

In this automated analysis process, the image was converted to a monochrome image (black and white) and the operator calibrated the grey level so that the section background staining was regarded zero. This was done in every section. The optical density was measured in the cell cytoplasm as mean grey, being equal to the sum of the optical density divided by the number of pixels measured, where sum of optical density is the sum of the calibrated optical density values for each pixel within the measured object. In addition to the mean grey, the immunoreactive area was measured in each cell. The cell immunoreactivity content (CIC) was calculated as the mean grey for the cell, multiplied by the immunoreactive area in μm^2. The data from each field were tabulated, computed and statistically analysed automatically.

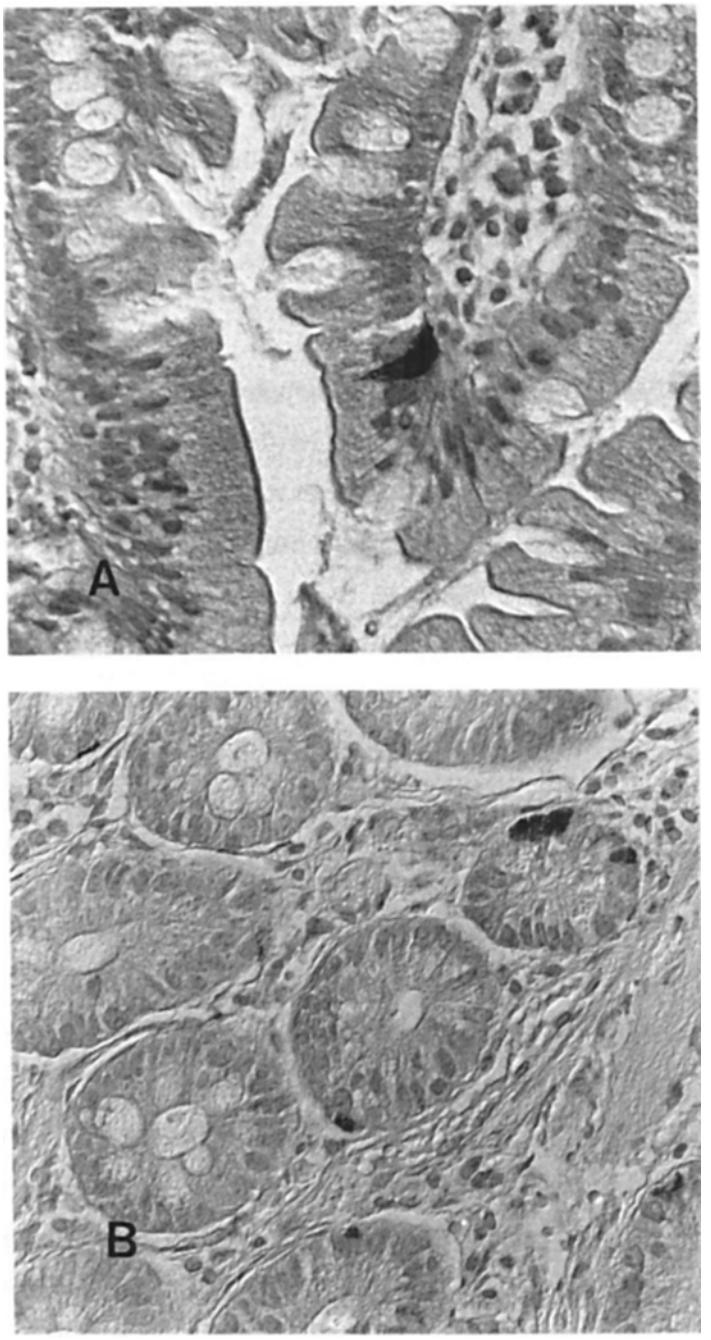

Fig.1. Secretin-immunoreactive cells in the villi (A), and gastrin/CCK-immunoreactive cells (B) in the crypts of proximal duodenum of a mouse. X350.

Results

Immunohistochemistry

Secretin- (Fig. 1a), gastric inhibitory polypeptide (GIP)-, gastrin/CCK- (Fig. 1b), somatostatin- and serotonin-immunoreactive cells were found both in the crypts and in villi, though they were more numerous in the crypts-except for secretin-immunoreactive cells, which occurred mostly in the villi. No glucagon- or motilin-immunoreactive cells could be detected in the examined proximal part of the mouse duodenum. The endocrine cells varied in shape, from flask- to basket-shaped and somatostatin-immunoreactive cells often showed a basal process. There was no immunostaining when the primary antibody was replaced by non-immune rabbit serum or when the primary antisera were pre-incubated with the corresponding antigen. Pre-incubation of the primary antibody with a structurally related antigen had no effect on the immunostaining.

Computerized image analysis

The results of image analysis of different endocrine cell types are summarized in Table 2. It seems that the most abundant cell is serotonin-immunoreactive cell type followed by gastrin/CCK-immunoreactive cells. The numbers of secretin-,GIP- and somatostatin-immunoreactive cells are almost the same.

DISCUSSION

In a previous report (2) the cell secretory index (CSI) was suggested as a parameter reflecting the endocrine cell peptide/amine content, which is a summation of cellular peptide/ amine synthesis and release. CSI represents volume of the cellular immunoreactive secretory granules. The cellular peptide/amine content is not only reflected by the immunoreactive secretory granule volume, but also by the intensity of immunostaining. In the present

Table 2. The number of various endocrine cell types per mm^3 of epithelial cells, the cell secretory index (CSI) mm^3 per cell and cell immunoreactivity content (CIC) in the duodenum of mice (mean±SE)

Endocrine cell type		Number of cells	CSI	CIC
Secretin	Crypts	3060±498	81.4±11.5	
	Villi	5078±314	161.6±81.8	
	Total	4036±284	84.8±7.7	5334.8±3002.6
GIP	Crypts	6911±443	77.8±4.6	
	Villi	5314±428	71.3±4.2	
	Total	6111±343	75.4±3.3	2294.5±1429.4
Gastrin/CCK	Crypts	10503±782	111.4±7.3	
	Villi	9481±638	130.8±8.5	
	Total	9979±427	121±5.8	1924.2±1633.5
Somatostatin	Crypts	6460±264	83.3±6.1	
	Villi	5000±353	80.8±4.7	
	Total	5737±178	83.3±5.1	6441.3±3336.3
Serotonin	Crypts	44626±3075	142.5±2.3	
	Villi	23246±769	151.6±7.6	
	Total	34801±1901	142.2±3.5	1447.2±108.6

study the CIC was suggested to be a more accurate parameter, reflecting the cellular peptide/amine content. In this parameter both the area and intensity of immunostaining are taken into account. It is worth noting, however, that the optical density measured here is a relative value, being related to the background staining of the section as zero. There are several possible sources of error in measuring the optical density of immunostaining: those caused by

immunostaining and those caused by the equipment used. Use of different dilutions of the first, second and third layers, or with DAB, as well as using different incubation times or temperatures, would result in different staining intensities. Furthermore, amplification, when ABC method is used, can vary if different kits, different biotinylated IgGs, or different incubation times with avidin and biotinylated peroxidase are used. Moreover, the optical density measurements are affected by the image brightness caused by the illumination of the microscope and by the operating parameters of the CCD-video camera. Being aware of all these pitfalls and by standardizing all these factors, optical density measurements and CIC can be useful in studies on endocrine cells, where comparison between the cell secretory contents in different groups is required.

The present study revealed five endocrine cell types in the proximal duodenum of the mouse. These cell types are similar in appearance and distribution to those described in the human duodenum (4,13). The finding that somatostatin-immunoreactive cells were more frequent in the crypts disagrees with an earlier report that they occurred mostly in the villi (6).

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