Pancreatic endoproteases and pancreatic secretory trypsin inhibitor immunoreactivity in human Paneth cells

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SUMMARY Normal and metaplastic gastrointestinal mucosa obtained at surgical resection were studied by light microscopy, using the unlabelled antibody enzyme method for immunohistochemical staining of lysozyme, pancreatic endoproteases, and pancreatic secretory trypsin inhibitor (PSTI). Paneth cells in the mucosa of normal small intestine, gastric mucosa with intestinal metaplasia, and colonic metaplastic mucosa were found to contain anionic trypsin, cationic trypsin, lysozyme, and PSTI immunoreactivity, but not chymotrypsin and elastase immunoreactivity. Normal gastric and colonic mucosa and some goblet cells in the small intestine showed positive PSTI immunoreactivity but no endoprotease immunoreactivity. The presence of immunoreactive trypsin and immunoreactive PSTI in the Paneth cells, which are of secretory type, probably indicates an important extrapancreatic source of these proteins rather than a storage of endocytosed material.

The Paneth cells originally described by Schwalbe in 1872¹ and later by Paneth in 1888² are normally found at the base of the crypts of Lieberkühn in the duodenum, jejunum, and ileum. Paneth cells are also found in metaplastic areas in gastric or colonic mucosa.³ They are ultrastructurally similar to secretory cells and like the pancreatic acinar cells, with apically located large cytoplasmatic granules. Among proposed properties and functions are the abilities to secrete immunoglobulins and lysozyme into the gut lumen and to eliminate metals from the mucosa into the luminal contents.⁴

Using an immunohistochemical technique, we found a trypsin like immunoreactivity in the Paneth cells in the duodenum, the small intestine, and in metaplastic areas in the gastric mucosa in man.⁵ The purpose of this investigation was to further characterise the trypsin like immunoreactivity and to determine if the Paneth cells also contain other pancreatic endoproteases as well as the pancreatic secretory trypsin inhibitor (PSTI).

Material and methods

TISSUE SAMPLES

The material comprised different resection specimens taken during operation from various types of surgical cases. The specimens were fixed in 10% buffered formalin and were imbedded in paraffin. The following types of mucosa were represented: normal mucosa from stomach, duodenum, jejunum, ileum, and colon; colonic mucosa from cases with ulcerative colitis; colonic mucosa containing glands with Paneth cell metaplasia; gastric mucosa of intestinal metaplastic type; and, in addition, colonic adenoma with Paneth cell metaplasia.

SPECIFIC MATERIAL

DEAE-Sephadex, Sepharose 4B, and prepacked PD 10 columns were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Trasylol was provided by Bayer AG, West Germany. Swine antirabbit IgG, peroxidase and antiperoxidase (PAP), and normal swine serum were obtained from Daco Immunochemicals, Copenhagen, Denmark. Di-iso-propyl fluorophosphate (DFP) was obtained from Sigma Chemicals (St Louis, United States of America).

Pancreatic juice was obtained from patients who had had the main pancreatic duct drained after pancreatic surgery. The pancreatic juice was collected in tubes on ice and frozen within two hours at -20°C . Human cationic trypsin and human PSTI were purified at the laboratory.^{6,7}

ANTISERA

Rabbit antisera against cationic trypsin, chymotrypsin, elastase 2, and PSTI were produced at the laboratory.^{5,7,8} Specific rabbit antihuman lysozyme serum was obtained from Professor Carl-Bertil Laurell, Malmö, Sweden.

AFFINITY PURIFICATION OF ANIONIC TRYPSIN ON SEPHAROSE CONJUGATED TRASYLOL

Trasylol (150 mg) was coupled to 15 g cyanogen bromide activated Sepharose 4B, according to the manufacturer's instructions. Pancreatic juice (50 ml) from a patient with a high concentration of anionic trypsinogen and only a small concentration of cationic trypsinogen was applied to the sepharose linked trasylol column ($2.5 \times 10\text{ cm}$), equilibrated with Tris hydrochloric acid buffer 0.01M, containing 0.1M sodium chloride at pH 8. After thorough washing with the starting buffer, until absorbance at 280 nm reached zero, the anionic trypsinogen was eluted with 0.01M formic acid containing 0.2M sodium chloride at pH 3.0. The fractions containing trypsinogen were desalted immediately on PD-10 columns, equilibrated with 0.001M hydrochloric acid, and lyophilised. The purified anionic trypsinogen showed only one protein band on agarose gel electrophoresis at pH 8.6: after autoactivation this band has a slightly less anionic mobility. The anionic trypsin was inactivated with DFP by adding 10 μl DFP (1M/l) in iso-propanol to 1 ml of anionic trypsin (1 mg/ml) in hydrochloric acid 0.001M/l. After the addition of DFP the pH was adjusted to 7.0 by adding 100 μl Tris buffer, 1M/l.

Antiserum against human anionic trypsin(ogen) was produced by immunising rabbits with human anionic trypsin inactivated with DFP and emulsified with Freund's adjuvant. Immunoelectrophoresis

against human pancreatic juice before and after activation showed one strong anionic precipitate, corresponding to anionic trypsinogen and trypsin. Sometimes a faint precipitate was seen, corresponding to cationic trypsin(ogen), probably indicating a cross reaction between the two trypsin, as has been reported previously.⁹

IMMUNOADSORPTION OF RABBIT ANTISERA AGAINST HUMAN ANIONIC AND CATIONIC TRYPSINS

Human cationic trypsin (8.8 mg) inactivated by DFP was conjugated to 1 g of Sepharose 4B, and 15 mg similarly inactivated human anionic trypsin was coupled to 1.5 g of Sepharose 4B, according to the manufacturer's instructions. Two separate columns, $0.9 \times 7\text{ cm}$ and $0.9 \times 11\text{ cm}$, were equilibrated in a glycine buffer, 0.1M/l, containing sodium chloride 0.15M/l, ethylene diamino tetra-acetate 0.005M/l, and sodium nitrate 0.02% at pH 6.8. Rabbit antiserum against human anionic trypsin (25 ml) was applied to the sepharose cationic DFP-trypsin column. The column was then washed with the same glycine buffer. The fractions containing protein were pooled and applied to the sepharose anionic DFP-trypsin column. The column was washed with the same glycine buffer until absorbance at 280 nm was below 0.1. Then the specific antibodies against human anionic trypsin were eluted with a glycine buffer, 0.1M/l, containing sodium chloride 0.15M/l, sodium thiocyanate 3.5M/l, ethylene diamino tetra-acetate 0.005M/l, and sodium nitrate 0.02% at pH 7.3. The absorption at 280 nm of the effluent was measured and the fractions containing antibody were pooled and dialysed against water.

The antiserum against human cationic trypsin was processed in the same way; first being filtered through the sepharose-anionic DFP-trypsin column, with the effluent from this column then being applied to the Sepharose-cationic-DFP-trypsin column. After washing with the starting buffer to an absorption at 280 nm of less than 0.1 the specific antibodies against human cationic trypsin were eluted with the buffer described above. The fractions containing antibodies

Table Pancreatic endoproteases and PSTI immunoreactivity in Paneth cells in investigated specimens with positive immunohistochemical staining

	Anionic trypsin	Cationic trypsin	PSTI	Chymotrypsin	Elastase
Normal mucosa:					
Duodenum	4/4	4/4	4/4	0/4	0/4
Jejunum	4/4	4/4	4/4	0/4	0/4
Ileum	4/4	4/4	4/4	0/4	0/4
Metaplastic mucosa:					
Stomach	4/4	4/4	4/4	0/4	0/4
Colon (ulcerative colitis)	5/6	5/6	5/6	0/6	0/6
Adenoma (Paneth cell metaplasia)	1/1	1/1	1/1	0/1	0/1

Fig. 1 Precipitation patterns obtained by immunoelectrophoresis of pancreatic juice with (A) rabbit anticationic trypsin serum, (B) rabbit antianionic trypsin serum, and (C) rabbit anti-PSTI serum. Arrow denotes application points.

Fig. 2 Normal jejunal mucosa. Lysozyme shown within Paneth cells by immunohistochemical staining. $\times 160$.

Fig. 3 Normal jejunal mucosa. Complete blocking of staining reaction using absorbed antisera against anionic trypsin. $\times 160$.

Fig. 4

Fig. 6

Fig. 5

Fig. 4 Gastric mucosa with focal intestinal metaplasia. Anionic trypsin shown within Paneth cells by immunohistochemical staining. (Haematoxylin and eosin.) $\times 160$.

Fig. 5 Gastric mucosa of fundus type with chronic gastritis and severe intestinal metaplasia. PSTI shown within Paneth cells (arrow heads) as well as in normal glands (arrow) by immunohistochemical staining. (Haematoxylin and eosin.) $\times 160$.

Fig. 6 Normal jejunal mucosa. Anionic trypsin shown within Paneth cells by immunohistochemical staining. $\times 160$.

Fig. 7 Normal jejunal mucosa. Cationic trypsin shown within Paneth cells by immunohistochemical staining. $\times 160$.

were pooled and dialysed against water.

The immunoabsorbed specific rabbit antibodies against human anionic and cationic trypsins were checked by immunoelectrophoresis against human pancreatic juice. The antibodies were used in dilutions made from a stock solution of 2 mg/ml.

IMMUNOHISTOCHEMICAL METHODS

The tissue samples were stained with haematoxylin and eosin and analysed with light microscopy. The peroxidase-antiperoxidase (PAP) method described by Sternberger *et al*¹⁰ with the slight modification already mentioned,¹¹ was used for localisation of the different pancreatic endoproteases, PSTI, and lysozyme. The antisera were used in serial dilutions 1/100 to 1/2000. Controls were performed with each new staining series using the antisera after immuno-sorbent absorption with their appropriate antigens and non-immune rabbit serum. Swine antirabbit IgG

Fig. 8 Normal jejunal mucosa. Anionic trypsin shown within Paneth cells by immunohistochemical staining. $\times 640$.

and peroxidase complexes were used in the dilutions of 1/50. All reagents were applied for 30 minutes at room temperature in a humidified chamber.

Results

After immunoabsorption of the anionic and cationic trypsin antisera no cross reactivity between them was seen, and only one distinct precipitation line was seen on immunoelectrophoresis for each antiserum (Fig. 1).

Fig. 9 Normal duodenal mucosa. PSTI shown in Paneth cells as well as goblet cells by immunohistochemical staining. (Haematoxylin and eosin.) $\times 1600$.

Fig. 10 Colonic mucosa in a case of ulcerative colitis with intestinal metaplasia containing Paneth cells. Anionic trypsin shown within Paneth cells by immunohistochemical staining. $\times 160$.

Fig. 11 Colonic adenoma containing Paneth cells. Anionic trypsin shown within Paneth cells by immunohistochemical staining. (Haematoxylin and eosin.) $\times 160$.

Fig. 12 Colonic adenoma containing Paneth cells. PSTI shown within Paneth cells by immunohistochemical staining. (Haematoxylin and eosin.) $\times 160$.

Fig. 9

Fig. 11

Fig. 10

Fig. 12

The Table shows the results of the immunohistochemical staining. The Paneth cells were identified on the basis of histological criteria and by their immunohistochemical staining for lysozyme (Fig. 2). A positive brown staining for the two trypsins and for PSTI was evident in Paneth cells of various locations. Control sections with normal rabbit sera or antisera previously absorbed against their appropriate antigens yielded negative results. (Fig. 3). No pancreatic chymotrypsin or elastase immunoreactivity could be shown in any control specimen.

STOMACH

In normal gastric mucosa no trypsin immunoreactivity was identified. In areas of intestinal metaplasia with Paneth cells a distinct trypsin immunoreactivity of both anionic and cationic types was identified in the Paneth cells (Fig. 4). The Paneth cells also contained PSTI immunoreactive material (Fig. 5). PSTI immunoreactive material was also seen in some areas of normal mucosa.

DUODENUM, JEJUNUM, AND ILEUM

In all specimens analysed a distinct positive anionic and cationic trypsin immunoreactivity was seen in the Paneth cells, both in the basal and apical parts of the cells (Figs. 6, 7, 8). PSTI immunoreactivity was seen in some Paneth cells and also in goblet cells in the basal parts of the glandular crypts of Lieberkühn (Fig. 9).

COLON

In the normal colonic mucosa a positive PSTI immunoreactivity was seen mainly in the basal part of the crypts. In the normal colonic mucosa no Paneth cells were identified, and no pancreatic endoprotease immunoreactivity was seen. In metaplastic areas of the mucosa in cases with ulcerative colitis, containing Paneth cells, positive anionic and cationic trypsin as well as PSTI immunoreactivity were identified within the cells (Fig. 10). In one case of adenoma with Paneth cell metaplasia distinct anionic and cationic trypsin immunoreactivity were identified (Fig. 11). PSTI immunoreactive material was also stored in various parts of the adenoma (Fig. 12).

Discussion

In a recent study using immunohistochemical methods,⁵ we reported on trypsin like immunoreactivity in the human Paneth cells in normal mucosa and in metaplastic areas of gastric mucosa. In the present study it was necessary to eliminate the cross reacting antibodies from each antiserum as the two trypsins show some cross reactivity. This was accomplished using an immunoabsorption technique

with the two isolated antigens bound in a solid phase. Applying the respective specific antibodies, we showed that the trypsin like immunoreactivity in the Paneth cells was of anionic and cationic types. The positive brown staining was completely abolished in the controls using the specific antisera previously absorbed with their appropriate antigens, further confirming the specificity of the reaction for anionic trypsin and cationic trypsin.

The Paneth cells of all tissues studied were easily identified according to their histological criteria and by their immunochemical staining for lysozyme. In addition to Paneth cells of metaplastic and normal types, immunoreactive PSTI was seen in areas of normal gastric and colonic mucosa and in some goblet cells in the basal parts of the crypts of Lieberkühn in the small intestine. No immunoreactive trypsin was identified in the goblet cells. Thus immunoreactive trypsin was always accompanied by immunoreactive PSTI, fitting the general concepts of Laskowsky,¹² tissues that contain a protease also always contain the appropriate inhibitor. Further direct isolation and characterisation of these immunoreactivities will probably clarify the presence of PSTI in some cells without apparent trypsin content.

A common observation in published reports is the ultrastructural resemblance between Paneth cells and the acinar pancreatic cells.⁴ Several findings also point to a functional resemblance. Paneth cells are of secretory type. Pilocarpine has been shown to increase the secretion from these cells.¹³ Trasylol, a potent trypsin inhibitor, increases the granule size in the Paneth cells.¹⁴ Studies in hamsters have shown that pancreatic duct ligation was followed by degeneration of exocrine pancreas and caused a hypertrophy of Paneth and goblet cells.¹⁵ The duodenal mucosa in patients suffering from chronic pancreatitis showed an appreciable increase in the number of Paneth cells.¹⁶ These data, as well as our proof that both trypsin immunoreactive material and PSTI immunoreactive material are present in the same type of cells, argue the case for a production of trypsin and PSTI in the Paneth cells. The findings of both immunoreactive anionic and cationic trypsin, as well as their appropriate inhibitor PSTI in Paneth cells, from a physiological and pathophysiological point of view, show that the Paneth cells are probably an important extrapancreatic source of these enzymes and give a further indication of the resemblance between Paneth cells and acinar pancreatic cells.

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