Impact of familial amyloid associated polyneuropathy on duodenal endocrine cells

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Abstract

Duodenal endocrine cells in 11 patients with familial amyloid associated polyneuropathy (FAP) were compared with those in 12 healthy volunteers by means of immunohistochemistry and morphometry. The total endocrine cell content, determined by the argyrophilic reaction and chromogranin A immunoreactivity, was significantly reduced in FAP patients compared with controls. There was a significant reduction in the serotonin, cholecystokinin/gastrin, and secretin immunoreactive cell content. A decreased cell content was also noted for somatostatin and gastric inhibitory polypeptide immunoreactive cells but this was not statistically significant. Amyloid deposits were noted in seven of the 11 biopsy specimens from FAP patients, but otherwise the duodenum was histologically normal in both groups. The reduction in endocrine cell content was not correlated with the degree of amyloid deposit in the duodenum. These findings indicate that patients with FAP have reduced intestinal endocrine cells. This does not seem to be related to amyloid deposits in the mucosa or to villous or crypt abnormalities. The observed changes in endocrine cells may contribute to the development of intestinal motility dysfunction and maldigestion in these patients.

(Gut 1994; 35: 1413-1418)

Amyloidosis represents a group of diseases characterised by the deposit of amyloid fibrils in various tissues. The protein precursor differs in various forms of amyloidosis. In several forms of familial amyloid polyneuropathy (FAP), mutated transthyretin (prealbumin) is the main fibril component. In Swedish FAP patients transthyretin is characterised by methionine at position 30 (met³⁰) instead of valine¹ as in FAP patients type I reported from Portugal² and Japan.³ FAP seems to occur throughout the world – cases have been reported in the USA,⁴ Ireland,⁵ China,⁶ Germany,⁷ France, Spain and Greece.⁸

In most patients, the initial clinical manifestation of FAP is a sensorimotor polyneuropathy starting in the legs and later affecting the arms.⁹ The autonomous nervous system is also affected, especially in more advanced stages of the disease. Symptoms include orthostatic hypotension, anhidrosis, sexual impotence, heart arrhythmia, and disturbance of gastrointestinal motility.⁹⁻¹¹

Instances of severe constipation, sometimes accompanied by nausea and vomiting, are often the initial gastrointestinal symptoms. With time the constipation is relieved by bouts of diarrhoea that subsequently become continuous.^{11 12}

The course of the disease is steadily progressive, and life expectancy has been reported from Sweden to be approximately nine years from the onset of the symptoms.⁹ The cause of death is usually intercurrent infection and extreme malnutrition.¹¹ As most transthyretin is produced by the liver,¹³ liver transplant has been performed, with encouraging results so far, on patients with FAP-met³⁰ mutation and steadily progressive disease.^{13 14}

Intestinal neuroendocrine peptides have a central role in the regulation of gastrointestinal motility, secretion, and ion transport.¹⁵⁻¹⁷ Several different modes of action have been described for these – that is, as paracrine/endocrine hormones, neurotransmitters, and neuromodulators.¹⁸ Since severe gastrointestinal disturbance in both motility and absorption are common in FAP, it is possible that the neuroendocrine system is affected in these patients.

This study aimed to investigate the endocrine cells in the duodenum of FAP patients and to compare the findings with those in healthy controls. The duodenum was chosen because it contains a large number and variety of endocrine cells.¹⁹

Methods

PATIENTS

Duodenal biopsy specimens from 11 patients with FAP (seven men and four women; mean age 47; range 28–70 years) were available for investigation. Nine patients had gastrointestinal symptoms (Table I). In all patients the diagnosis was based on the histological finding of amyloid deposits in skin or rectal mucosa, and electromyographical abnormalities consistent with peripheral polyneuropathy of the axonal type. In addition, the diagnosis was verified by DNA analysis^{20 21} for mutation of transthyretin in position 30 (FAP-met³⁰). Clinical data were obtained from the patient's medical records and are summarised in Table I. Two patients were homozygous for the FAP-met³⁰ gene.

Twelve healthy volunteers (six men and six women; mean age 39; range 22–71 years) who had given their written consent to the study served as controls. These volunteers had no gastrointestinal symptoms. The difference in

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Accepted for publication
31 January 1994

TABLE I Clinical data of patients with familial amyloid associated polyneuropathy

Patient no	Sex	Age (y)	FAP met ³⁰ mutation	Polyneuropathy (y)	Gastrointestinal dysfunction (y)	Gastrointestinal symptoms	Autonomic nerve dysfunction	Peripheral polyneuropathy
1	M	60	Homoz	9	7	Nausea, vomiting; intermittent diarrhoea; sig-moideostomy	Urinary retention; urethral catheter	Severely disabled, confined to wheelchair
2	M	47	Heteroz	10	6	Continuous diarrhoea; GEA	Urinary hesitation; faecal incontinence; autonomic heart dysfunction	Walk short distances with two crutches
3*	F	43	Heteroz	6	5	Continuous diarrhoea; sig-moideostomy; GEA	Urinary hesitation	Walk short distances with two crutches
4†	F	47	Heteroz	10	3	Intermittent diarrhoea	Urinary hesitation	Walk with one crutch
5‡	M	36	Heteroz	5	5	Continuous diarrhoea	Urinary hesitation; faecal incontinence	Confined to wheelchair
6	M	57	Heteroz	8	2	Intermittent diarrhoea; sig-moideostomy	Orthostatic hypotension; urinary retention; uretral catheter	Severely disabled; confined to wheelchair
7	F	70	Heteroz	19	0	None	None	Walk short distances with two crutches
8	M	42	Heteroz	7	3	Nausea, vomiting, continuous diarrhoea	Urinary hesitation; faecal incontinence; autonomic heart dysfunction	Walk with two crutches
9	F	38	Heteroz	8	3	Intermittent diarrhoea	Faecal incontinence; autonomic heart dysfunction	Walk short distances with two crutches; otherwise confined to wheelchair
10	M	52	Heteroz	1	0	None	Autonomic heart dysfunction	Sensory disturbances in the legs
11	M	28	Heteroz	2	2 Month	Intermittent diarrhoea	Sexual impotence	Sensory disturbances in the legs

Homoz=homozygous for FAP met³⁰ mutation; Heteroz=heterozygous. *Patient died aged 45 years; †died aged 52 years; ‡died aged 39 years. GEA=gastro-entero-anastomosis.

age between patients and controls was not statistically significant.

The investigation was approved by the local committee for medical ethics, at the University of Umeå.

UPPER GASTROINTESTINAL ENDOSCOPY

Gastroduodenal endoscopic examinations were performed after an overnight fast. Endoscopic biopsy specimens were obtained from the pars descendens duodeni, distal to the papilla of Vater, either by means of a Watson biopsy capsule attached to the gastro-scope or by biopsy forceps. The presence of solid residue in the stomach was recorded in the endoscopy reports, and regarded as an indication of gastric retention. Controls were subjected to an identical gastroduodenal endoscopic procedure.

MATERIAL AND HISTOPATHOLOGICAL TECHNIQUE

Biopsy specimens were fixed in 4% buffered formaldehyde overnight, embedded in paraffin, and cut at 5 µm. Sections from both patients and controls were stained with

haematoxylin and eosin, van Gieson stain, and Grimelius's silver nitrate method.²² The presence of amyloid deposits was determined by polarisation microscopy of sections stained with alkaline Congo red.

MORPHOMETRY

Mucosa

A 10×10 mm square grid (Zeiss) was inserted in a ×12.5 eyepiece. The baseline of the grid was aligned with the base of the crypts in three sections cut perpendicular to the mucosa from each individual. The number of villi and crypts in an area 1 mm² was counted using the ×10 objective. The villus height was measured with a 1 mm scale inserted in a ×12.5 eyepiece and a ×10 objective.

Amyloid deposits

The amount of amyloid deposit was assessed by Haugh point count technique.²³ Thus, a 121 point quadratic lattice (Zeiss, 10×10 mm, 19 mm φ, no 434008) was inserted in an ×12.5 eyepiece. The lattice was aligned so that it covered the whole thickness of the mucosa, muscularis mucosa, and the part of submucosa available in each section. The number of amyloid deposits in the mucosa, muscularis mucosa, and submucosa situated at the grid points were counted, and at least 1000 points were counted in three sections from different levels of each biopsy specimen. Amyloid deposits were expressed as the percentage of the ratio between the number of points situated over amyloid deposits and the total number of grid points situated over the previously mentioned structures.

NEUROENDOCRINE CELLS

Immunohistochemistry

Each section was deparaffinised, hydrated, immersed in 0.01% hydrogen peroxide in Tris

TABLE II Detailed account of the antisera used

Antisera raised against*	Working dilution	Code no	Source
Chromogranin A†	1:1500	LK2H10	Baehrynger, Mannheim, Germany
Serotonin†	1:1600	Mas055, clone YC5/45 HLK	Seralab Ltd, Crawley Down, Sussex, UK
Gastrin‡	1:5000	1336-1	Milab, Malmö, Sweden
Synthetic somatostatin	1:1600	054-P	Dako A/S, Glostrup, Denmark
Porcine secretin	1:800	R1-7	WY Chy, Rochester, NY, USA
Bovine gastric inhibitory peptide (GIP)	1:1000	R65	Novo Nordisk A/S, Bagsvaerd, Denmark
Porcine glucagon§	1:1200	R-78101-B2	Milab
Porcine glucagon§	1:1000	A56S	Dako
Porcine glucagon**	1:1600	K964	Novo Nordisk A/S
Bovine neurotensin	1:1600	M-8205	E Theoderson, Dept of Clinical chemistry, Karolinska Hospital, Stockholm, Sweden
Synthetic pure porcine motilin††	1:1200	R-1105	N Yanaihara, Shizuoka, Japan

*All the antisera raised in rabbit except †, which are monoclonal antibodies. ‡Specific for CCK/gastrin C-terminus. §Specific for glucagon N-terminus and cross reacts with both pancreatic glucagon and entrogucagon. **Specific for glucagon C-terminus reacts only with pancreatic glucagon. ††Specific for C-terminus.

TABLE III Distribution and amount of amyloid deposition in biopsy specimens from pars descendens duodeni of FAP patients

Patient no	Amyloid deposition			Amyloid deposit (% of tissue)	Endocrine cell area (µm ² /mm ²)*	Gastric stasis	BMI (kg/m ²)
	Mucosa	Musclaris mucosa	Submucosa				
1	No	No	No	0	25 000	Present	18.5
2	No	No	No	0	13 000	Present	21.0§
3	Yes	Yes	Yes	7.1	325	GEA	15.5
4	Yes	Yes	Yes	7.1	120	Absent	18.3
5	Yes	Yes	Yes	8.4	1 430	Present	15.7
6	No	No	No	0	50	Present	21.2
7	No	No	No	0	500	Absent	20.6
8	Yes	Yes	Yes	34.7	8 250	Present	19.3§
9	Yes	Yes	Yes	2.7	13 140	Present	20.1
10	Yes	Yes	Yes	8.5	15 280	Absent	23.7
11	Yes	Yes	Yes	5.5	9 240	Absent	NA

*Endocrine cell area as detected by the argyrophilic reaction, expressed as µm²/mm² of epithelial cells (controls: mean (SD)=14 870 (4710)). BMI=body mass index. †Patients with nephrotic syndrome and oedema. NA=not available.

HCl buffer (pH 7.4) for 10 minutes to inhibit endogenous peroxidase activity, washed three times with TRIS buffer, and treated with 1% bovine serum albumin for 30 minutes. Incubation took place overnight with the following antisera: anti-chromogranin A, anti-serotonin, anti-gastrin, anti-somatostatin, anti-secretin, anti-gastric inhibitory polypeptide (GIP), anti-glucagon, anti-neurotensin, and anti-motilin (for details concerning the antisera used see Table II). The sections were then processed by the avidin-biotin complex method (ABC, Dako A/S, Glostrup, Denmark). Briefly, sections were incubated with biotinylated swine anti-rabbit IgG or biontinyated anti-mouse IgG diluted 1:200 for 30 minutes. They were then incubated with the avidin-biotin-peroxidase complex diluted 1:100 for 30 minutes. Development of the section was performed in 50 ml Tris buffer containing 10 µl of 30% H₂ O₂ and 25 mg diaminobenzidin-hydrochloride (DAB).

The specificity controls were similar to those described previously.²⁴ Briefly, negative controls were obtained by using normal rabbit serum (Tris buffer in case of monoclonal antibodies) in place of the primary antiserum and by preincubating antiserum with an excess of

the corresponding or structurally related antigen (75–100 µg/ml diluted antiserum) for 24 hours at 4°C. Positive controls were obtained by processing sections known to contain the neuroendocrine peptide studied with each staining experiment.

Morphometry

This was performed by the IBAS-Micro version 2.0 program, using the IBAS automatic image analysis system (Kontron Electronic, Munich, Germany) connected to a Carl Zeiss microscope, type axiophot. The epithelial cell area was determined by two threshold levels. To discriminate between epithelial cells (including endocrine cells) and background and lamnia propria, the upper limit and lower limit were determined by interactive setting. This was performed by moving the cursor on the tablet to the X and Y positions. The cursor key #5 was pressed once to freeze the lower threshold and again to freeze the upper threshold. The endocrine cell area was estimated by using the interactive image editor, which erased the outside region. This was done by drawing a continuous line around the endocrine cells contour and erasing outside structures. For each endocrine cell type, 40 randomly chosen fields of villous and crypts from four sections, 30 µm apart, were used from each individual. Each field seen in the monitor represents 0.16 mm² of tissue. The quantity of endocrine cells was expressed as µm²/mm² of the lining epithelium. Sections were coded and the investigator who performed the measurements, did not have the code key. All measurements were performed by one investigator (ME).

STATISTICAL ANALYSIS

Differences between groups were analysed by means of the Wilcoxon rank sum test; correlation was determined by Spearman's rank test. p Values below 0.05 were regarded as statistically significant.

Results

ENDOSCOPY

The presence of solid residue in the stomach was reported in seven of the FAP patients but in none of the healthy controls. All patients with gastric stasis had gastrointestinal symptoms, whereas one without this had intermittent constipation and diarrhoea (Tables I and III).

HISTOPATHOLOGICAL EXAMINATION

Histopathological examination of specimens from FAP patients and healthy controls showed a normal histological structure. The average number of villi in FAP patients was 3.5/mm² (range, 3–4) and in controls 3.7/mm² (range, 3–5). The average number of crypts in FAP patients was 8/mm² (range, 6–10) and in

Figure 1: Amyloid deposits in the pars descendens duodeni of a FAP patient. The deposits look green (white in the photomicrograph) when examined in polarised light. The deposits can be seen in the mucosa (M), muscularis mucosa (MM), and submucosa (SM). (Alkaline Congo red stain, ×150.)

Figure 2: Argyrophil cells in the villous of a healthy control (A) and of a FAP patient (B). (Grimelius stain, ×450.)

controls it was 7.5/mm² (range, 7–10). The average height of the villi in FAP patients was 0.9 mm (range, 0.8–1) and in controls this was 0.9 mm (range, 0.7–1.1). No significant differences were found between patients and controls with regard to the numbers of villi, crypts, or the villous height. Amyloid deposits were noted in seven specimens from FAP patients (Fig 1), details of the localisation and quantity of these deposits are given in Table III. No correlation was found between the degree of amyloid deposition in the duodenum and the duration of disease.

ENDOCRINE CELLS

Argyrophilic (Fig 2), chromogranin A, serotonin (Fig 3), cholecystokinin (CCK)/gastrin C-terminus, somatostatin (Fig 4), secretin, and GIP immunoreactive cells were observed in the pars descendens duodeni of both FAP patients and controls. Argyrophilic, chromogranin A, and serotonin immunoreactive cells were found in both the villi and crypts. CCK/gastrin and secretin immunoreactive cells were confined almost to the villous epithelium. Somatostatin, and GIP immunoreactive cells were mainly localised within

crypts. Glucagon, neurotensin, and motilin immunoreactive cells were not detected in either controls or FAP patients.

Specificity controls showed that the antisera immunostained endocrine cells in the small intestine of the rats. Immunostaining was abolished completely after preincubation with the corresponding peptide. Preincubation of the antisera with the structurally related peptides had no effect on the immunostaining. Replacing the antisera with normal rabbit serum or Tris buffer gave no staining.

The endocrine cell content, as demonstrated by argyrophilic cells and chromogranin A immunoreactive cells, was significantly reduced in FAP patients compared with controls ($p<0.01$ and 0.001 respectively). There were significantly fewer serotonin, CCK/gastrin, and secretin immunoreactive cells in FAP patients compared with controls ($p<0.01$, 0.05 , and 0.001 respectively). Decreased cell contents, which were not statistically significant, were noted for somatostatin and GIP immunoreactive cells (Table IV). No significant correlation was found between age and endocrine cell content, either for patients or controls. No correlation was found between the body mass index (BMI) and the endocrine cell content (Table III). No significant correlation was found between the total endocrine cell content and the presence or the degree of the amyloid deposits.

Discussion

Gastrointestinal absorption, secretion, coordinated peristalsis, blood flow, and the

Figure 3: Serotonin immunoreactive cells in the crypts of a healthy control (A) and of a FAP patient (B). (ABC technique without counterstaining, ×150.)

Figure 4: Somatostatin immunoreactive cells in the crypts of a healthy control (A) and of a FAP patient (B). (ABC technique and counterstained with haematoxylin, $\times 350$.)

immune response are regulated by nervous and endocrine/paracrine mechanisms.^{15 16} The nervous control is exerted by central ‘extrinsic’ (parasympathetic, sympathetic, and sensory) as well as by local ‘intrinsic’ neurons.¹⁶ The degree of extrinsic control varies between different parts of the gastrointestinal tract. Thus, the parasympathetic system seems to control the motor activity of the oesophagus and stomach as well as the gastric secretion, but has no influence on the intestinal functions.¹⁶ Sympathetic innervation inhibits intestinal secretion, gut motility, and blood flow. The intrinsic innervation represents the majority of the nervous elements in gastrointestinal tract with the neuropeptides as the predominating neurotransmitters.¹⁶

The peptidergic neurons act as motor, vasomotor, secretory, or sensory neurons.¹⁵ Several peptides of the intrinsic nervous system regulate the intestinal motility. Thus, substance P has a contractile effect and neuropeptide Y (NPY), vasoactive intestinal polypeptide (VIP), and galanin have a relaxing

effect on the intestinal smooth muscles.¹⁶ The endocrine/paracrine regulatory system is composed of scattered cells among the epithelial cells of the mucosa. These cells are divided into 11 cell types depending on the peptide or biogenic amine they contain, as follows: secretin, gastrin, CCK, GIP, somatostatin, enteroglucagon, neurotensin, PP, PYY, motilin, and serotonin.^{19 25 26} These cell types have a special pattern of distribution in the gastrointestinal tract, which has a certain bearing on their functions. Thus, serotonin and somatostatin cells occur throughout the gut. Gastrin cells occur only in stomach. CCK, secretin, enteroglucagon, neurotensin PP, and PYY cells are found in the small intestine. Enteroglucagon, PP, and PYY cells are located in the large intestine.^{19 25 26}

It is noteworthy that the endocrine cells have several peptides/biogenic amines in common with peptidergic nervous system namely, somatostatin, gastrin/CCK, neurotensin, and serotonin. These cells exert their effect through secretion of their peptides into the blood stream (endocrine function) to be carried to the target organ or into the interstitial fluid (paracrine function) to diffuse to their site of action.¹⁹ Both the nervous and endocrine/paracrine regulating systems interact and integrate in regulating the previously mentioned functions of the gastrointestinal tract.¹⁵

In FAP patients abnormalities in the extrinsic and the classical part of the intrinsic innervation of the gut have been observed. Thus, amyloid infiltration and severe loss of myelinated nerve fibres in the celiac ganglion and vagus nerve have been found in FAP patients.²⁷ Furthermore, low level of catecholamines have been reported in familial amyloid intestine.^{28 29} Changes in the peptidergic nerve system in these patients are unknown. This is due to the difficulty in obtaining whole wall biopsy specimens from FAP patients, as their condition never calls for surgery.

In this study both the argyrophilic reaction and chromogranin A immunoreactivity were used as markers for the total number of endocrine cells, according to previous recommendations.³⁰ The endocrine cell content was significantly low in FAP patients compared with healthy controls. Of the endocrine cell types analysed, the serotonin, CCK/gastrin, and secretin immunoreactive cells were significantly reduced. It has been established

TABLE IV Mean (SD) area of crypt and villous epithelium occupied by endocrine cell types in FAP patients and healthy controls

Cell type	FAP patients				Controls			
	Crypt	Villous	Total	No	Crypt	Villous	Total	No
Argyrophil	8404 (8698)†	4270 (6146)	7904 (8276)‡	11	21212 (7984)	7395 (5009)	14870 (4705)	11
Chromogranin A	6714 (5421)*	4460 (5068)‡	5585 (4837)*	11	22132 (9410)	11939 (9492)	17361 (4369)	12
Serotonin	5452 (7883)‡	2014 (3021)†	4064 (5382)†	10	14209 (6885)	10187 (8178)	12765 (6389)	10
CCK/gastrin	0	507 (973)‡		10	0	3943 (2738)		11
Somatostatin	1625 (1817)	0		10	3581 (1706)	0		10
Secretin	0	186 (467)*		11	0	5955 (4500)		11
GIP	1817 (2705)	0		11	3492 (2072)	0		9

Endocrine cell area expressed as $\mu\text{m}^2/\text{mm}^2$ of epithelial cell area. Statistically different between FAP patients and controls; * $p<0.001$; † $p<0.01$; ‡ $p<0.05$. GIP=gastric inhibitory peptide; CCK=cholecystokinin; total=total endocrine cell area in crypt and villous epithelium.

that serotonin exerts both direct and indirect excitatory effects on the gut smooth muscles, where indirect effects are mediated by the cholinergic nerves. CCK has a stimulatory effect on antral and pyloric contraction and an inhibitory effect on the proximal stomach.^{16 18} In the small intestine, CCK initiates peristaltic activity and increases the efficiency of the peristaltic reflex.¹⁸ The reduction in these cells in FAP patients may lead to impairment of intestinal motility and the development of gastric stasis, promoting bacterial contamination and malabsorption.^{7 11 12} CCK and secretin stimulate the pancreatic secretion of enzymes and bicarbonates. The significant reduction observed in the area of these cells may contribute to the development of maldigestion and steatorrhea.^{11 12}

The reduction in endocrine cells did not correlate with the presence or the degree of the amyloid depositions. In addition, the villous and crypt structure of amyloid and healthy intestine was histologically similar. These findings indicate that the reduction in the endocrine cell area in FAP patients is not caused by amyloid deposits in the mucosa or villous and crypt changes.

Further studies are needed to establish whether the decrease in the endocrine cells observed here in patients with amyloid associated neuropathy is associated with decreased secretion of these hormones. This can be done by determination of the hormonal level in tissue extracts or counting the secretory granules in electron micrographs, or both. Furthermore, it could be of great value to correlate these findings to the abnormal gastrointestinal motility shown in FAP patients by different motility tests.

This study was supported by grants from the Swedish Medical Research Council (projects no 102 and 11240), the Åke Wibergs Stiftelse, the Medical Faculty, Umeå University, the Joint Committee for Medical Research, Northern Health Region, and the Swedish Medical Association.

Preliminary results were presented in abstract form in the 2nd International Symposium on Familial Amyloidotic Polyneuropathy and Other Transthyretin Related Disorders, held in Skellefteå, Sweden, from 1–3 June 1992; and in the first International Workshop on Modern Analytical Methods in Histochemistry, held in Salzburg, Austria, from 14–18 September 1992.

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