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Immunohistochemical localization of pepsinogen A and C containing cells in Barrett's oesophagus*

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Summary. No data are available on the localization of Pepsinogen A (PGA = PG I) and Pepsinogen C (PGC = PG II) positive cells in Barrett's epithelium. Endoscopic biopsy specimens were taken from the columnar epithelium from 23 patients ($n = 93$), and in addition from the cardia from eight healthy control subjects ($n = 38$). The tissue was stained by the immunoperoxidase technique with specific anti-pepsinogen antisera, and double immunostained for PGA and PGC. In the Barrett's epithelium PGA was found in 28 out of 93 biopsy specimens (30.1%) and PGC in 55 out of 93 (59.1%). Chief cells always stained both for PGA and PGC, while clear mucous cells were often PGA− and PGC+. PGA+ and PGC+ cells were found each in 100% of the biopsy specimens with fundic type epithelium, in 21.7% and 70.7% of biopsy specimens with junctional type, in 0% and 26.1% of biopsy specimens with specialized epithelium and in 12.5% and 43.5% of biopsy specimens with mixed junctional/specialized features respectively. Dysplastic epithelium stained always negatively with both anti-pepsinogen antisera. In most control cardia biopsy specimens PGA as well as PGC were demonstrable; occasionally clear mucous glands were PGA− and PGC+.

It is concluded that pepsinogen-containing cells can be accurately identified in the Barrett's epithelium; their presence seems related to the histological cell type. Identification of pepsinogen positive cells may contribute to a more accurate morphological classification of the Barrett's epithelium.

Key words: Barrett's oesophagus – Pepsinogen – Immunohisto-chemistry

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Introduction

Barrett's oesophagus is characterized by the presence of columnar cell-lined epithelium and classified histologically into three main types: junctional, gastric fundic and specialized columnar (Paull et al. 1976). According to this classification, chief cells and parietal cells are present in the gastric fundic type only. However, no detailed information is available on the presence or absence of pepsinogen A and C containing cells in the Barrett's epithelium.

The aim of our study therefore was to determine whether pepsinogen-containing cells can be identified in the Barrett's epithelium by means of specific anti-pepsinogen antisera and if so, is there any correlation between the presence of pepsinogen positive cells and the main type of Barrett's epithelium?

Materials and methods

Twenty-three patients (age ranging from 18–81 years) with circumferential type Barrett's oesophagus were studied. Endoscopic biopsy specimens ($n = 110$) were taken in a random fashion from the columnar cell-lined epithelium from non-ulcerated areas below the squamo-oesophageal junction and at least 1 cm above the gastro-oesophageal junction, determined by endoscopical assessment. In addition, in eight control subjects without gastrointestinal disease (age ranging from 43–79 years) biopsy specimens ($n = 38$) were taken from the cardiac area within 1 cm from the mucosal transition. Of the 110 biopsy specimens from the Barrett's epithelium 17 were excluded, because of the main presence of squamous epithelium; therefore 93 biopsy specimens remained for final analysis.

After sampling, the biopsy specimens were fixed for three hours in formol sublimate and processes as described elsewhere (Meuwissen et al. 1985). Serial sections were stained with haematoxylin and eosin (H and E), Periodic Acid-Schiff (PAS), PAS-Alcian Blue (pH 2.5), anti-pepsinogen A (anti-PGA = anti-PG I), anti-pepsinogen C (anti-PGC = anti PG II) and double immunostained (anti-PGA plus anti-PGC) (vide infra). Characteristics of the preparation of the anti-PGA antiserum (Meuw-

issen et al. 1985; Pals et al. 1985), as well as of the anti-PGC antiserum have been published (Pals et al. 1986). In brief: antibodies were raised in goats and in rabbits. Initial immunization was done by intramuscular injection of 1 mg PGA or PGC (in goats) or 0.3 mg PGC (in rabbits) in PBS, mixed with complete Freunds adjuvant. Booster injections were given at 4 weeks intervals, using 0.5 mg (goats) or 0.15 mg PG (rabbits) in incomplete Freunds adjuvant. After determination of titers in small samples, blood was taken 10 to 14 days after booster. Anti-PGA and anti-PGC were isolated by affinity chromatography, using pure PGA and PGC coupled to CNBr activated Sepharose-4B (Pharmacia, Sweden). The bound antibodies were eluted from the column with 3 M NaSCN, immediately deionized by gel filtration on Sephadex G25 (Pharmacia, Sweden) and lyophilized. Specificity of antibodies was confirmed by electrophoresis of homogenates of gastric mucosa after preincubation with the produced anti-PGA or anti-PGC and by ELISA, using PGA and PGC coated plates. Affinity purified anti-PGA from goat was conjugated with horse radish peroxidase (HRP; type VII; RZ=3.0; Sigma, St. Louis, MO, USA) by the two step glutaraldehyde method. Goat anti-human PGA-horse radish peroxidase was prepared from affinity-purified antibodies (Pals et al. 1985). PGA was demonstrated by a direct immunoperoxidase technique. After being deparafinized sections were incubated for 30 min with 0.3% H_2O in methanol to block endogenous peroxidase activity, washed in PBS (phosphate-buffered saline, pH 7.4) and preincubated with normal swine serum (1:10) for 10 min, followed by goat anti-human PGA-HRP for three hours (1:500 in PBS with 1% BSA=bovine serum albumin). Thereafter sections were washed in PBS (3 × 10 min) and stained with a freshly prepared DAB (diamino benzidine)– solution, containing 0.03% H_2O_2. Haematoxylin was used for counterstaining. For demonstration of PGC an indirect immunoperoxidase technique was used, the first steps being identical as for PGA. Rabbit anti-human PGC was applied for two hours (dilution 1:50 in PBS with 1% BSA). After washing (PBS, 3 × 10 min) sections were preincubated with normal goat serum (1:10), followed by swine anti-rabbit IgG (Dako, Denmark), conjugated with HRP (1:1000 in PBS with 1% BSA) and stained and counterstained as described above.

To obtain a blue instead of a brown reaction product, alkaline phosphatase (AP) conjugated goat anti-rabbit IgG (Sigma, St. Louis, MO, USA) was used as a second antibody step; alkaline phosphatase was revealed using naphtol AS–MX phosphatase as substrate and Fast Blue BB as coupling agent (both from Sigma, St. Louis, MO, USA) (Boorsma 1980; Mason et al. 1983). Control experiments for PGC were performed by incubation with normal rabbit IgG instead of rabbit anti-human PGC. For the exact identification of PGA and PGC within individual cells a double immunostaining technique was used (Mullink et al. 1986): sections were initially incubated with goat anti-human PGA–HRP (1:500), stained with DAB, followed by incubation with rabbit anti-human PGC (1:50) and goat anti-rabbit IgG–AP (1:50). In serial section (with anti-PGA, anti-PGC and anti-PGA/PGC double staining) it was shown that PGA containing cells always contained PGC, but not vice versa. Therefore it was concluded that the brown or brown-bluish staining cells in the double stained sections contained PGA as well as PGC, while the blue staining cells were only PGC positive.

In all experiments biopsy specimens from normal gastric fundic mucosa served as control tissue: the fundic mucosa always stained positively for PGA and PGC in chief cells and mucous neck cells; parietal cells, surface mucous epithelium and other cellular elements remained negative. Identical results for human fundic biopsy specimens were reported recently (Meuwissen et al. 1985). Biopsy specimens from the Barrett's epithelium were categorized as junctional, gastric fundic, specialized columnar (Paull et al. 1976) without previous knowledge of the results of the PG-incubation. In addition, a mixed junctional/specialized type was recognized. The presence of pepsinogen positive cells per biopsy specimens was scored as: occasional positive cell, several positive cells, many positive cells.

Results

The 38 biopsy specimens from the normal cardia showed either gastric fundic mucosa with some

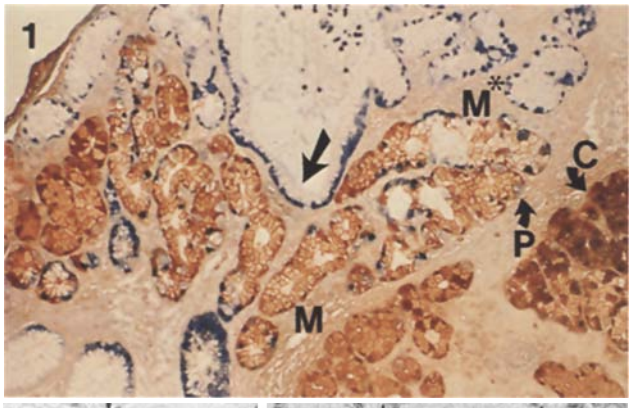
Fig. 1. Control human cardia. The majority of glands consists of brown staining mucous glands (PGA and PGC positive) (*M*). The larger cyst, lined with gastric surface epithelium (arrow) and the other mucous glands (*M**) are staining blue for PGC. Small number of chief cells (*C*) and parietal cells (*P*) are visible (anti-pepsinogen A and anti-pepsinogen C, immuno-double stain × 70)

Fig. 2 a. Control human cardia, showing gastric surface epithelium, clear mucous glands and fundic glands. The mucous glands are PGA-positive, showing a 'transparent' staining, while chief cells stain darker ('granular'). Parietal cells are negative (anti-pepsinogen A × 112). **b** Control human cardia, serial section. Similar results, except positive staining of the deeper part of the gastric pit (*arrow*). The intensity of the stain is weaker compared with PGA (anti-pepsinogen C × 112)

Fig. 3. Control human cardia. The deeper part of the gastric pit is positive for PGC (anti-pepsinogen C and alkaline phosphatase as 2nd step × 175)

Fig. 4. Junctional biopsy Barrett's epithelium, consisting of a large cystic gland and smaller glands, lined with gastric surface epithelium, staining positively at the base of the cell (*M*). In addition some fundic glands, with chief and parietal cells, are seen. The arrow indicates a PGA negative mucous gland which was PGC positive in the serial section (anti-pepsinogen A × 77)

Fig. 5 a. Mixed biopsy Barrett's epithelium with mainly junctional glands of the left and specialized intestinal glands on the right, with an island of squamous epithelium in the middle. The arrows indicate a small cluster of clear mucous glands, possibly containing a few parietal cells (× 59). **b** Detail of the area of **a**, indicated by the arrows. On the left the deeper part of a gastric pit (PGC positive) next to some glands. About 50% of the mucous cells are brown (PGA and PGC positive), while the other mucous cells are blue (PGC positive) (anti-pepsinogen A and anti-pepsinogen C, immuno-double stain × 385)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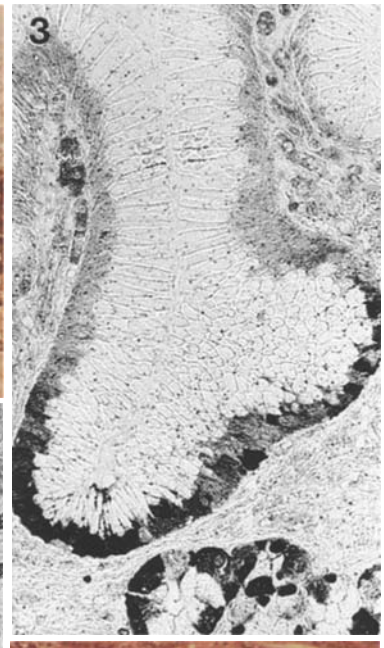
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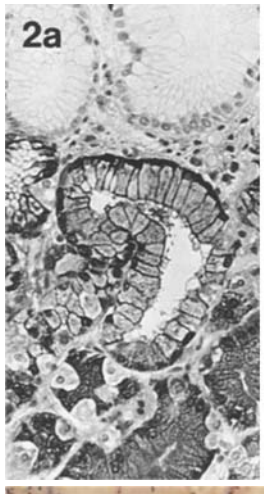
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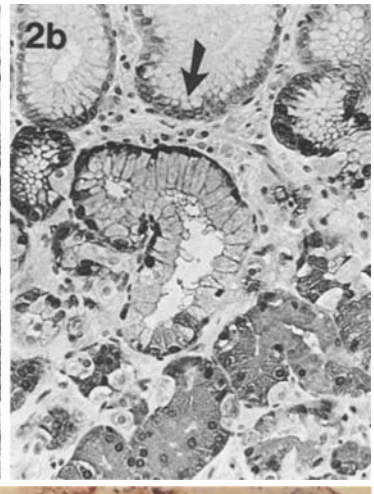
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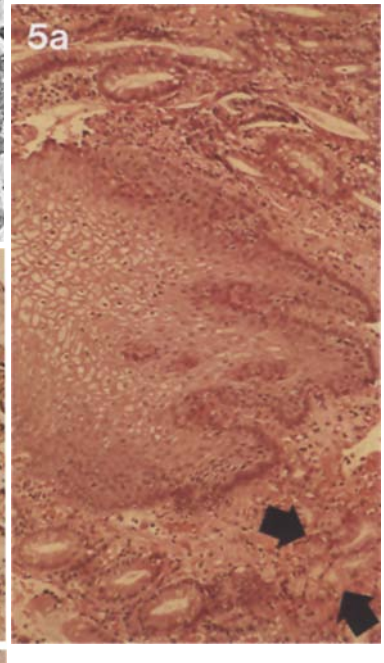
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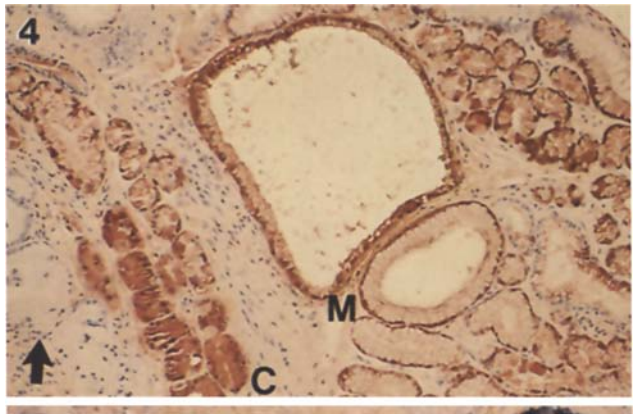
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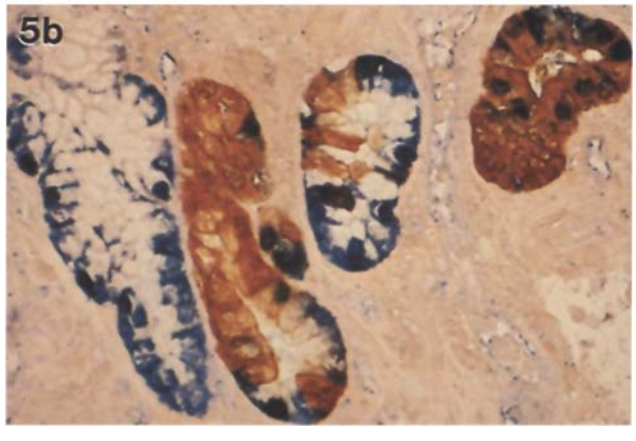
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clear mucous glands or gastric cardiac mucosa with mainly clear mucous glands, occasionally some cystic glands and a few glands of fundic epithelium. All mucous glands were PAS positive with an occasional Alcian Blue positive cell. The large majority of the biopsy specimens (35/38) showed in the glandular layer of the mucosa isolated PGA-positive glands or clusters of positive glands, while all biopsy specimens were PGC-positive. A representative biopsy specimen of normal human cardia stained with anti-PGA and anti-PGC is shown in Fig. 1. The positive glands were either of the gastric fundic or of the clear mucous type. The cytoplasm of the chief cell always stained strongly positive with anti-PGA and anti-PGC in a granular fashion, while the cytoplasm of the clear mucous cells stained rather faintly with a dark positive reaction at the base of the cell and around the nucleus (perinuclear area). Clear mucous glands were either PGA and PGC positive or PGA negative and PGC positive. In the normal cardiac mucosa no PGA and PGC negative glands were found. An example of the gastric fundic type mucosa with tortuous glands and a pronounced zone of mucous gland cells is shown in Fig. 2a and b. Parietal cells never stained with anti-PGA and anti-PGC nor did the gastric mucosal surface; however the deeper part of the gastric pit was usually positive for PGC (Fig. 3).

PGA was demonstrated in 28 out of 93 biopsy specimens (30.1%) with Barrett's epithelium and PGC in 55 out of 93 (59.1%). Occasional positive PGA cells were found in five biopsy specimens (5.4%), 10 (10.8%) showed several positive PGA cells, while 13 (13.9%) biopsy specimens were strongly positive. These figures were for PGC seven (7.5%), 21 (22.5%) and 27 (29.0%) respectively. The difference in numbers of PGA and PGC positively staining cells was never caused by chief cells which were always PGA and PGC positive, but only by the presence of PGA negative, PGC positive clear mucous cells. The pattern of pepsinogen staining of the gastric fundic type was not distinguishable from that of fundic glands in the normal cardia. Clear mucous glands were found mainly in the mucosa of the junctional and less in the mucosa of the specialized and mixed junctional/specialized type. The staining characteristics of the clear mucous glands in the junctional type was strikingly different, with regard to PGA and PGC. PGA was detectable in one-third of the junctional biopsy specimens and PGC in more than two-third (Table 1). An example of a junctional biopsy specimen with some fundic glands, stained for PGA, is shown in Fig. 4. It was not possible to predict

Table 1. Presence of pepsinogen-containing cells, related to main type Barrett's epithelium

	No.	Pepsinogen A	Pepsinogen C
Gastric fundic or Mixed fundic/junctional	13	13 (100 %)	13 (100 %)
Junctional	41	13 (31.7%)	29 (70.7%)
Specialized intestinal	23	0 (0 %)	6 (26.1%)
Mixed specialized/junctional	16	2 (12.5%)	7 (43.8%)
	93	28	55

positive pepsinogen staining in clear mucous glands by means of studying serial H and E or PAS-AB sections. In addition, it occurred that the majority of clear mucous cells in a submucosal gland was PGA and PGC negative, while only a minority of these glands was PGA and PGC positive or only PGC positive. However, when parietal cells were detected in the mucous glands (mixed junctional/fundic glands), staining was always positive for PGA and PGC. Biopsy specimens showing the specialized intestinal type or with features of severe intestinal metaplasia were always PGA negative and occasionally PGC positive, while mixed junctional/specialized biopsy specimens showed sometimes small clusters of PGA and PGC positive cells (Fig. 5a and b). The villar surface was always PGA and PGC negative. However in a rare instance some focal PGA and PGC 'lining' of the epithelium occurred. Features of mild and moderate dysplasia were found in several biopsy specimens of the junctional and specialized type; dysplastic epithelium however was always PGA and PGC negative.

Discussion

Several studies have demonstrated that Barrett's epithelium produces pepsinogens, for example, patients with a Barrett's oesophagus were stimulated with a secretagogue (Betazole) and oesophageal secretions were aspirated separately from the stomach and analyzed by agar gel electrophoresis (Mangla et al. 1985). Sonication of endoscopic biopsy specimens from Barrett's epithelium and staining for proteolytic activity has also shown that pepsinogens are present in the columnar-lined mucosa (Mangla et al. 1973, 1976; Mangla, Camp et al. 1980; Mangla 1985; Westerveld et al. 1986, 1987). Pepsinogen A appeared to be found most frequently in the fundic type and least frequently in the specialized type (Westerveld et al. 1987). The exact localization fo pepsinogen A and C in the

foveolar and/or glandular layer of the Barrett's epithelium is only possible however in immunohistochemical studies using specific antisera against human pepsinogens. To date no data have been published. Only few references deal with the normal cardia. PGC positive and negative clear mucous glands were detected in the vicinity of the gastro-oesophageal junction, depending on the overlaying epithelium and the place of sampling (Weinstein et al. 1977). In another study normal cardia glands stained only with anti-PGC (Samloff 1983). In a recent study, using the immunoperoxidase technique, no PGC positive glands were found in the cardiac mucosa; the study however was performed on a limited number of gastric specimens (Busby-Earle, Williams et al. 1986). From our study, also using the immunoperoxidase technique, it appeared that biopsy specimens from the normal cardia showed either gastric fundic type epithelium, staining in a positive fashion with anti-PGA and anti-PGC or as characteristic junctional epithelium with clear mucous glands and cystic glands. A considerable number of the glands stained with both anti-pepsinogen antisera, while a minority was PGA negative (Fig. 1). The intensity of staining was often rather weak however, which may be the expression of a high intracellular concentration of mucosubstances as well as of a low intracellular concentration of pepsinogens. Moreover, it is likely that the weak staining intensity can partly be explained by the fact that secretory granules in antral gland cells have a stainable core with anti-PGC antiserum, surrounded by a clear matrix without any PGC reactivity (Cornaggia et al. 1987). Chief cells biopsy specimens from controls as well as from patients with a Barrett's oesophagus contained always PGA and PGC and this appeared also to be true for mucous neck cells in the gastric fundic type.

Cornaggia has attempted to define the immunoelectron microscopical aspects of the different gastric mucosal cell types, with regard to their pepsinogen-content (Cornaggia et al. 1986, 1987). In our present study no attempt has yet been made to distinguish mucous neck cells from other clear mucous cells; this may be difficult with the immunohistochemical method, particularly in the junctional type of Barrett's epithelium. Whenever chief cells and/or parietal cells were found, a positive staining of PGA and PGC mucous cells also occurred. Such a distinction should therefore be made by electron immunocytochemistry. The main determinant for pepsinogen positive staining of clear mucous glands was the histological type of Barrett's epithelium. Glands in the mucosa of the gastric fundic type always stained for PGA and PGC but only occasionally stained in the mucosa of the specialized intestinal type. The largest variability was observed in the mucosa of biopsy specimens with features of the junctional type; in contrast with the normal cardia, clear mucous glands were often PGA and PGC negative. It is not clear whether the different staining characteristics of these mucous glands represent a spectrum of differential staining, otherwise normal glands or may hint at some "genetic" deregulation in the Barrett's epithelium. The normal surface epithelium of all three histological types almost always stained negatively for pepsinogens. Dysplastic epithelium was always negative for PGA and PGC, as were multiple biopsy specimens from the Barrett's epithelium of two patients with a Barrett's carcinoma (unpublished data). It is therefore not likely that pepsinogen antisera may be of value in routine clinical use to detect dysplasia in the Barrett's epithelium. It is also evident that the search for small clusters of fundic glands and chief cells will be greatly facilitated, thus contributing to a more accurate morphological classification of Barrett's epithelium. We have recently shown that isozymograms obtained from columnar cell-lined epithelium frequently differ from those in the gastric fundus (Westerveld et al. 1986, 1987). Morphological studies with isozymogen-specific monoclonal anti-pepsinogen antibodies may indeed confirm these observations.

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