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ORIGINAL ARTICLE

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Identification of Paneth cells in pyloric glands associated with gastric and intestinal mixed-type intestinal metaplasia of the human stomach

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Abstract We have proposed that intestinal metaplasia (IM) of the human stomach be divided into two types on the basis of cell differentiation status: a gastric and intestinal (GI) mixed type and a solely intestinal (I) type. In the GI mixed type, gastric (foveolar epithelial and pyloric gland cells) and intestinal (goblet, intestinal absorptive, and Paneth cells) phenotype cells coexist in the same intestinalized gastric glands in various combinations and degrees. Consequently, intestinalized gastric glands are hybrids. Although we have described the rare appearance of Paneth-like cells in pyloric glands of GI mixed-type IM, the absence of an appropriate Paneth cell marker leaves room for doubt as to their true character. The purpose of this study was to clearly identify Paneth cells in pyloric glands in IM lesions using a new Paneth cell marker, a polyclonal antibody human defensin (HD)-5, raised against HD-5, which is included in granules of Paneth cells. A total of 105 gastric samples (4 biopsy and 101 surgical resected specimens) were examined. In only nine cases (8.6%), the antibody allowed demonstration of Paneth cells in pyloric glands in GI mixed-type IM, confirming our previous finding. Analysis of the proliferative cell (P) zone indicated that a common stem cell might generate both GI phenotype cells by upward and downward migration. No Paneth cells were found above the P zone. The results suggest that the stem cells show abnormal cell differentiation in IM lesions but preserve their normal direction of migration.

Keywords Paneth cell · Pyloric gland · Intestinal metaplasia · Cell differentiation marker · Defensin 5

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Introduction

Intestinal metaplasia (IM) has been extensively studied as a possible premalignant condition in the human stomach [6, 30, 38]. However, the pathogenesis of IM development and its relationship to gastric cancers remain to be unequivocally confirmed. Recently, *Helicobacter pylori* (HP) infection has been established to be a major cause of gastroduodenal ulcers, chronic atrophic gastritis, and probably IM development [2, 4, 5]. Because IM is rather heterogeneous, a number of classifications have been proposed [6, 16, 30]. Most have focused on the similarities of IM to small or large intestinal epithelial cells morphologically, histochemically, or enzymatically [30].

We have proposed a unique classification based upon the cell differentiation status using both gastric and intestinal (GI) cell phenotypic markers [13]. With this classification, IM is divided into two major types; a GI mixed type and a solely intestinal (I) type. In the I type, three kinds of intestinal phenotype cells, goblet, intestinal absorptive, and Paneth cells, completely replace the gastric glands. In the GI mixed type, gastric phenotype cells are found sharing the same gastric gland with intestinal phenotype cells in various combinations. In many cases of the GI mixed type, atrophied pyloric glands are present under the intestinalized gastric pits. Although they are often diminished in size and decreased in cell number, they are apparently continuous to the upper intestinalized gastric pits across the proliferative cell (P) zone [13]. In very rare cases, we have found small numbers of Paneth-like cells, with prominent eosinophilic granules in their cytoplasm in such glands. Although

Paneth cells are frequent components of complete type IM [16], their presence in pyloric glands is controversial. Because no appropriate specific Paneth cell marker has hitherto been available, the question of the real identity of Paneth-like cells apparent on hematoxylin and eosin (H&E) staining has remained unanswered. Fortunately, we now have an antibody against human defensin-5 (HD-5) [22, 23, 25], which is one of the defensins – antibiotic peptides expressed in human and animal myeloid and some kinds of epithelial cells. The antibody almost exclusively reacts with HD-5 and allows clear detection of Paneth cells observed in the normal human small intestine and in the intestinal metaplastic glands [23].

The present study was aimed at demonstrating the presence of Paneth cells in pyloric glands associated with GI mixed-type IM using the antibody HD-5 in addition to class III mucin staining. Moreover, using MIB-1 immunohistochemistry, particular attention was paid to the distribution of both Paneth and pyloric gland cells relative to the P zone.

Materials and methods

All cases examined in this study were collected from the pathology file of Aichi Cancer Center Hospital and affiliated hospitals. A total of 105 gastric samples were studied. Four were biopsy specimens taken for diagnosis and 101 were from surgically resected carcinomas. The 78 male and 27 female patients ranged in age from 26 years to 77 years, with a mean of 59 years. The surgical material was separated into one or more specimens of carcinoma and surrounding non-neoplastic mucosa. Fifty-seven cases examined were fundic, and 48 were pyloric. As positive controls for Paneth cells, surgically resected human duodenal and jejunal samples were also examined. All materials were fixed in 0.1 M phosphate buffered 10% formalin, dehydrated through a graded alcohol series, cleared in xylene, and embedded in paraffin.

The generation of the HD-5 antibody was described previously [22, 23]. Briefly, recombinant human intestinal defensin-5 (rHD-5), biosynthesized by the baculovirus-insect cell expression system (Clontech, Palo Alto, Calif.), was coupled to ovalbumin as a carrier molecule and then injected mixed with physiological saline and Freund's adjuvant into female New Zealand White rabbits. Two boosts at 30-day intervals were similarly administered. The antibody titer of rabbit immune serum was determined using an enzyme-linked immunosorbent assay (ELISA). The specificity of the antibody was verified by means of dot blot and western blot assays [22, 23].

IM was evaluated and divided into two types, GI mixed type and I type, in H&E sections according to our previously described criteria [13]. The degree of IM was estimated as mild, moderate, and severe according to the proportion of the section occupied: less than one-third, over one-third to two-thirds, and over two-thirds, respectively. Serial sections of representative cases were then cut at 4 μm, mounted on APS-coated slides (Matsunami glass, Tokyo), and stained in the following order: first section, mucin histochemistry of paradoxical concanavalin-A (PCA) staining to identify pyloric gland cells; second, immunohistochemistry for HD-5 to identify Paneth cells; third, double staining of these two to demonstrate coexistence of Paneth and pyloric gland cells; and fourth, immunohistochemistry for Ki-67 antigen to analyze the P zone.

The precise staining procedures and the characteristics of PCA were as described previously [15, 33, 37]. Through PCA staining, mucins detected in the alimentary tract can be classified into two main types: (1) class III mucins in mucous neck cells, pyloric gland cells, and Brunner's gland cells; and (2) class II mucins in surface mucous cells, goblet cells, and the surface coat of intestinal absorptive cells. The class II mucins lose, whereas class III retain or increase their reactivity with a reduction step interposed between oxidation and concanavalin-A staining.

For HD-5 immunohistochemistry, the indirect immunoalkaline phosphatase method was employed [23, 26]. In brief, after deparaffinization and dehydration, sections were treated sequentially with normal goat serum to block nonspecific antibody binding, rabbit anti-HD-5 antibody (1:2000), and affinity-isolated alkaline phosphatase (ALP)-conjugated goat anti-rabbit immunoglobulins (Dako, Glostrup, Denmark). ALP-binding sites were visualized using a fuchsin substrate system (Dako, Carpinteria, Calif.) according to the manufacturer's instructions.

Double staining of PCA and HD-5 immunohistochemistry was achieved as follows: after PCA staining was performed using diaminobenzidine (DAB) as the chromogen, sections were thoroughly washed with phosphate buffered saline (PBS) and then incubated with the HD-5 antibody. The procedures after the primary antibody reaction were as described above. By this double staining, pyloric gland cells were stained brown and Paneth cells were stained blue.

For immunohistochemistry of the Ki-67 antigen as the proliferative marker, the avidin-biotin peroxidase complex (ABC) method was employed as described earlier [11, 17] with the mouse monoclonal antibody MIB-1 (Immunotech, Marseille, France) at a dilution of 1:100. Sections were lightly counterstained with hematoxylin for microscopic examination.

Results

Immunostaining of Paneth cells in the adult human small intestine

Although the specificity of the HD-5 antibody binding in histological sections has already been examined [23], it was again tested using adult human duodenal and jejunal tissue sections. Clear immunoreactivity limited to the cytoplasm of Paneth cells was evident at the bottom of the duodenal (Fig. 1a, b) and jejunal crypts (data not shown).

Identification of Paneth cells in IM lesions

First, evaluation of the IM type in H&E sections of all cases revealed that seven (6.7%) had no accompanying IM, while the other 98 (93.3%) demonstrated metaplasia to various degrees. Of the total, 51 (48.6%) were predominantly accompanied by the GI mixed type, 43 (41.0%) by the I type, and the other four (3.8%) were approximately equally with both types to the same degree. In only 9 out of 105 cases (8.6%), a few Paneth-like cells with intracytoplasmic eosinophilic granules appeared to be present among the mucous cells in pyloric glands (Fig. 2a). The mucous cells were PCA positive, while the Paneth-like cells showed immunoreactivity with the HD-5 antibody (Fig. 2b), confirming their true Paneth nature.

Double staining of pyloric gland cells and Paneth cells

Figure 2c illustrates the obvious coexistence of pyloric gland cells (brown) and Paneth cells (blue) in the same

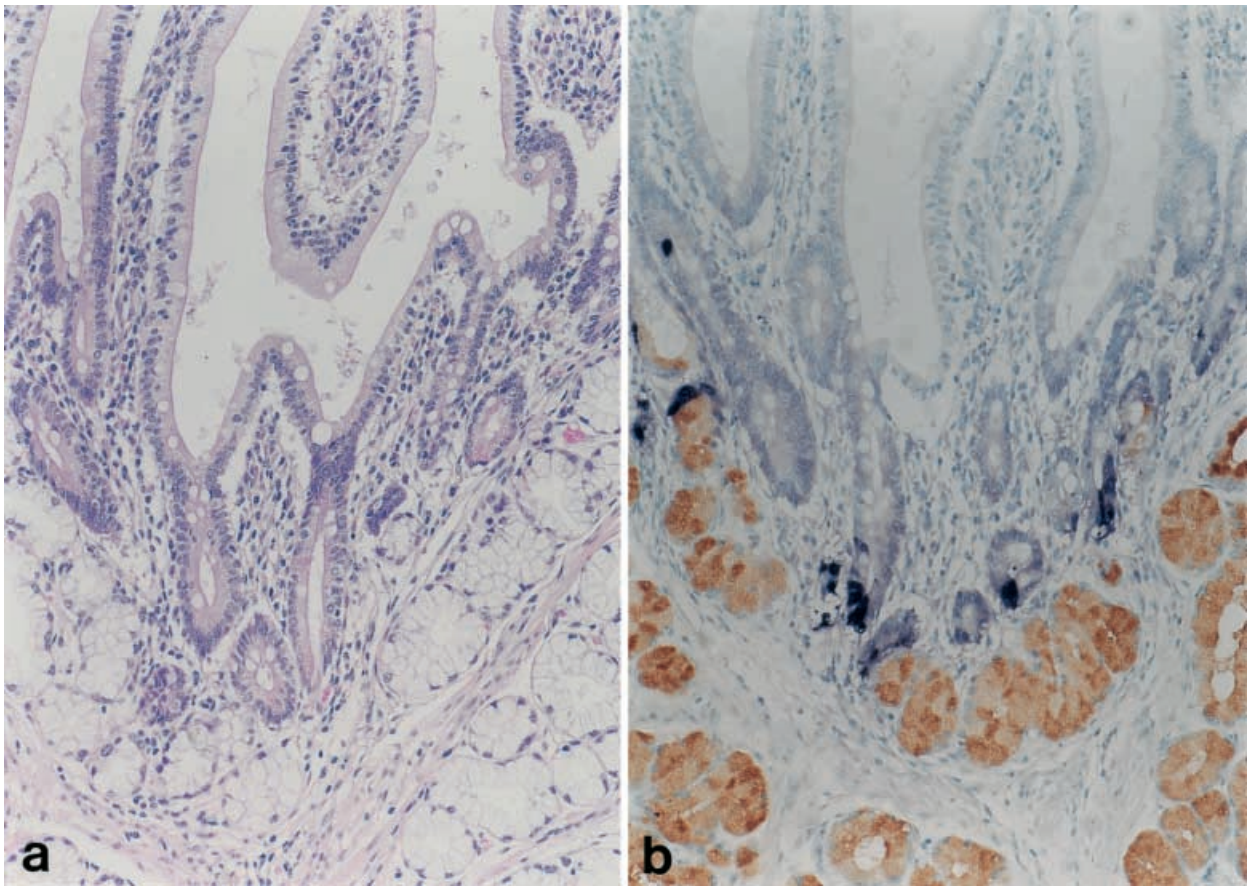

Fig. 1 Semi-serial sections through normal duodenal mucosa. **a** Paneth cells are present at the bottom of each crypt, while Brunner's gland cells are found both in the lamina propria and in the submucosa across the lamina muscularis mucosae. (hematoxylin and eosin, original magnification, ×40). **b** Paneth cells express immunoreactivity with the anti-human defensin (HD)-5 antibody, while Brunner's gland cells are positive for paradoxical concanavalin-A staining (double staining for HD-5 and class III mucins, original magnification, ×40)

Table 1 Clinicopathological data for cases with Paneth cells in pyloric glands. *P* pyloric, *F* fundic, *F with P* fundic gland with pseudopyloric gland, *IM* intestinal metaplasia, *GI* gastric and intestinal mixed-type IM, *I*, solely intestinal-type IM, *Pa* Paneth cells, *sig* signet-ring cell carcinoma, *well diff.* well differentiated adenocarcinoma, *mod. diff.* moderately differentiated adenocarcinoma, *por* poorly differentiated adenocarcinom, *N.D.* not determined because of the difficulty of correct estimation due to the small amount of sample

Case no.	Age (years)	Gender	Biopsy/operated	P/F	Degree of IM	Type of IM	Histology of cancer	Number of GI glands coexisting with pyloric gland cells and Pa cells/total number of GI glands (incidence) [number of Pa cells in each pyloric gland (average)]
1	69	Female	Biopsy	P	Moderate	GI>I	No cancer	N.D.
2	65	Male	Biopsy	P	Severe	GI≅I[a]	No cancer	N.D.
3	52	Female	Operated	F with P	Severe	GI>I	Well diff.	3/48 (6.3%) [3, 11, 15 (9.7)]
4	56	Male	Operated	P	Severe	GI≅I[a]	Well diff.	2/52 (3.8%) [5, 7 (6.0)]
5	52	Male	Operated	P	Moderate	GI>>I	Mod. diff.	3/48 (6.3%) [2, 3, 13 (6.0)]
6	54	Female	Operated	F with P	Severe	GI>I	Mod. diff.	6/65 (9.2%) [2, 6, 10,10, 12, 15 (9.2)]
7	52	Female	Operated	F with P	Severe	I>>GI	Well diff.	1/30 (3.3%) [6 (6.0)]
8	72	Male	Operated	P	Severe	GI>>I	Well diff.	6/110 (5.5%) [1, 2, 5, 6, 9, 10 (5.5)]
9	75	Female	Operated	P	Moderate	GI>>I	Por – sig	4/48 (8.3%) [1, 2, 5, 5 (3.3)]

[a] That is to say, nearly equal

pyloric gland of a GI mixed-type IM. Paneth cells were also observed at the bottom of I-type IM glands without pyloric glands (data not shown). Table 1 summarizes clinicopathological and histological data for the nine cases. There were no apparent common characteristics, although the degree of IM was severe in most, and there was a tendency for a link with GI mixed-type IM rather than the I type. The number of Paneth cells found in one

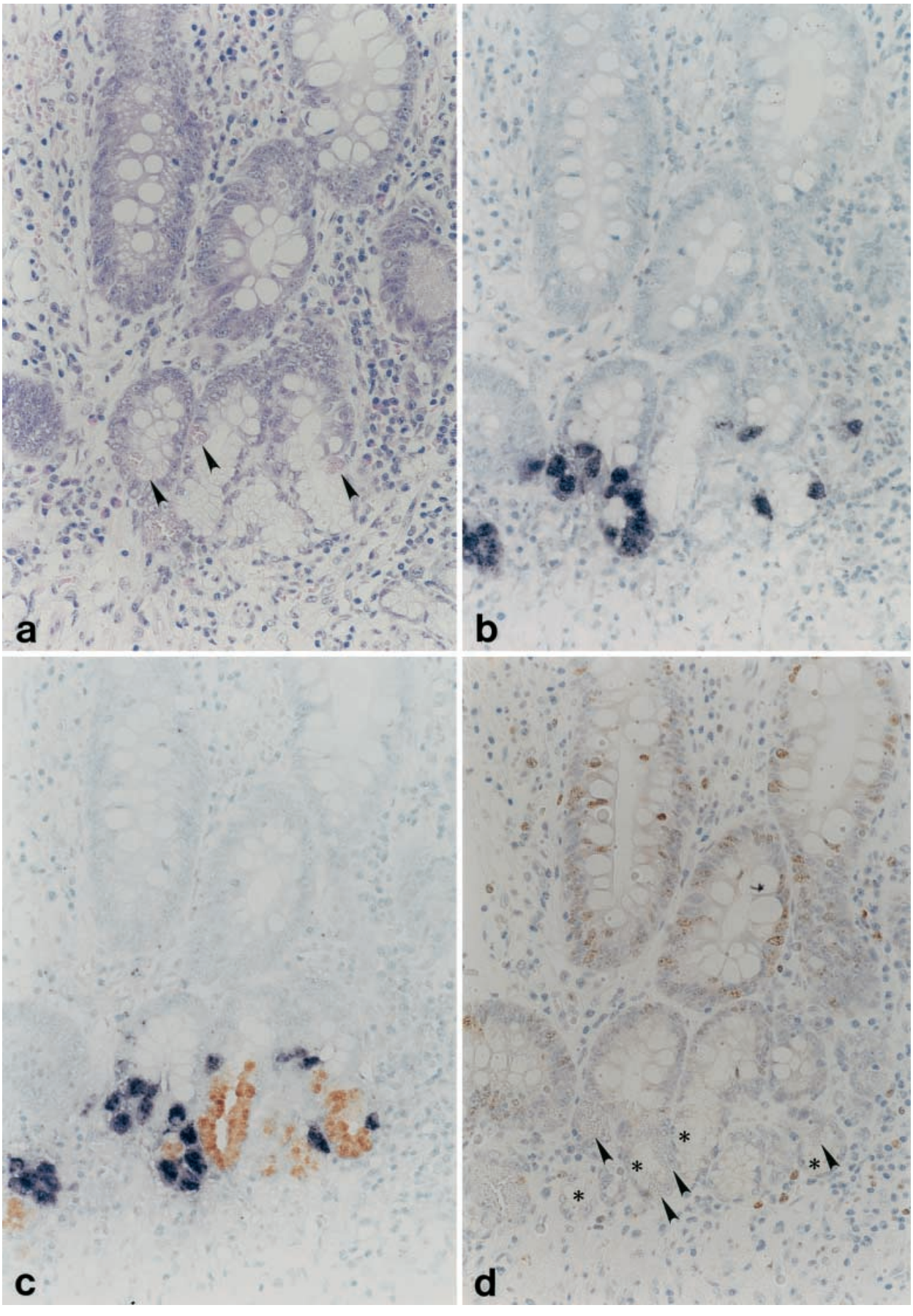

Fig. 2a–d Legend see page 18

pyloric gland was less than 15, with an average of 6.6. The numbers and incidences of GI mixed-type IM glands with coexistence of pyloric gland cells and Paneth cells were also estimated, confirming the rarity of this phenomenon (Table 1).

Detection of the P zone

The P zone was located in about the lower third of the I-type IM glands as in normal small intestine (Fig. 2d). Paneth cells were exclusively present underneath this region. In GI mixed-type IM glands with coexisting pyloric gland cells and Paneth cells, proliferating cells were perceived between the upper intestinalized gastric pits and the underlying pyloric glands in which Paneth cells were present.

Discussion

Our new classification of IM into GI mixed and I types is based upon the cellular differentiation status of IM glands using various cellular differentiation markers [13]. Hitherto, the IM classification has been mainly focused just on the appearance of intestinal phenotypic cells, but in our previous study, we first clearly demonstrated that a good many gastric phenotypic cells may remain, admixed with intestinal phenotypic cells in various combinations and degrees in the same IM glands, and we therefore termed this GI mixed-type IM. This allowed us not only to introduce the notion that IM might be due to an abnormal stem cell differentiation, but also to consider IM subtypes as not an independent entity but a sequence of pathological states, with gradual change from the stomach to the intestine. Moreover, this has made it possible to categorize lesions that did not fit well into the common Filipe's [6] or Kawachi's [16] classifications.

The present immunohistochemical study revealed that Paneth cells may indeed be present in pyloric glands in rare cases of GI mixed-type IM. Thus, the antibody against HD-5, a human homologue of alpha-defensin that was first cloned from murine small intestine and subsequently demonstrated to be localized in Paneth cells [22, 23], clearly showed positive staining in cells with eosinophilic granules. It is well known that Paneth cells possess lysozymal activity in their cytoplasmic granules [29]. Although such enzyme activity is not specific to Paneth cells, a histochemical method using substrates might be useful for demonstration of Paneth cells in some cases. However, pyloric gland cells also have lysozymal activity in their cytoplasm. Thus, the HD-5 antibody, which exclusively binds to HD-5 molecules included in Paneth cell granules [23], has major advantages for identification purpose.

Of the nine cases in which Paneth cells were observed in pyloric glands, seven had gastric carcinomas (Table 1). In one case (case 8), the GI mixed-type IM with Paneth cells in pyloric glands was located just beneath the carcinoma which was of well differentiated, intestinal type but without obvious Paneth cell differentiation (data not shown). Moreover, no correlation between carcinoma location and distribution of IM, including pyloric glands with Paneth cells, was demonstrated in the other six cases. Although it has been suggested that development of intestinal-type carcinomas could have a close interrelationship to IM, especially of incomplete type [6, 30, 38], our data do not support this hypothesis.

Paneth cells in an IM lesion were observed both in metaplastic pseudopyloric glands within the fundic mucosa and in the pyloric glands proper. We have proposed that the histological characteristics of IM shown in the fundic mucosa are similar to those of pyloric gland mucosa if fundic glands are accompanied by pseudopyloric gland metaplasia [13]. Our present results are compatible with our previous findings in this context.

It is believed that stem cells (multipotent progenitor cells) are present in the P zone in the isthmus region of the gastric glands, giving rise to all of the various cell types by differentiation so that consequently gastric glands are monoclonal in the adult stage [14, 18, 21, 34, 35]. In the environment of a normal gastric gland, undifferentiated stem cells undergo complex bipolar migration from the isthmus either upward or downward [24]. In fact, only the cells that are destined to be surface mucous cells move upward. Those cells that are committed to becoming pyloric, cardiac, or the three types of fundic gland cells and endocrine cells migrate downward. In the crypts of the small intestine, however, stem cells would be expected to be present in the P zone at the bottom of the crypts. In the normal intestinal gland, cells that will become absorptive, goblet, and endocrine cells move up, but only these differentiating into Paneth cells migrate lower than the P zone. The latter frequently appear in the intestinal metaplasia and are occasionally found in gastric adenomas or colorectal polyps [27, 28, 32, 36], while being exceedingly rare in gastric adenocarcinomas [3, 10, 19, 20]. The underlying mechanism remains to be clarified. In our cases, all Paneth cells were below the P zone, mimicking the normal small intestinal crypts. Sugihara et al. [31], Akamatsu et al. [1] and Fujimori et al. [7] revealed an organoid differentiation of intramu-

◀ **Fig. 2** Semi-serial sections through intestinalized gastric mucosa in which Paneth cells are apparent in pyloric glands (case 6). **a** A few Paneth-like cells with intracytoplasmic eosinophilic granules are observed within pyloric glands in a gastric and intestinal (GI) mixed type intestinal metaplasia (IM) gland (*arrowheads*; hematoxylin and eosin, original magnification, ×40). **b** Blue coarse granules, which are immunoreactive for anti-human defensin (HD)-5 antibody, are obvious in the cytoplasm of Paneth cells (immunostaining for anti-HD-5, original magnification, ×40). **c** Paneth cells (*blue*) and pyloric gland cells (*brown*) are admixed in the same intestinalized gastric gland (double staining for HD-5 and class III mucin, original magnification, ×40). **d** Both Paneth cells with granules (*arrowheads*) and pyloric gland cells (*asterisk*) are present under the proliferative cell zone composed of MIB-1 positive cells (MIB-1 immunohistochemistry, original magnification, ×40)

cosal signet ring cell or diffuse-type carcinomas, including Paneth cells, with disturbance of the tissue architecture once tumor cells undergo submucosal invasion. The available data would suggest that the appearance of a Paneth cell, in pyloric glands of mixed-type IM might be the consequence of abnormal differentiation of stem cells, while the normal cell migration pattern is preserved. Since epithelial cell differentiation and migration of gastric glands is thought to be closely linked, the reason why only the former is disturbed is not clear. Recently, epithelial-mesenchymal interactions have attracted attention due to an alteration in miscellaneous conditions and their role in organogenesis [8, 9, 12]. The question of whether the organization of an IM lesion might be influenced by interstitial cells clearly warrants further study.

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