and GLP-1 are typical of the intestinal endocrine cell markers.[15–18] We first immunohistochemically analyzed the expression of these endocrine cell markers in six distinct sites of the normal gastrointestinal tract (Table 2). We then analyzed the endocrine phenotypes in the same 1030 fundic and 2004 pyloric glandular ducts for the number per gland and incidence (% positive glands/total glands).

Double immunohistochemical staining of gastrin and GLP-1

Double staining of gastrin and GLP-1 was achieved as described previously.[30] Sections were immunostained with GLP-1 first by the peroxidase method using AEC (AEC high substrate chromogen system, DakoCytomation, Glostrup, Denmark) as a chromogen. Slides were thoroughly washed with Tris-buffered saline (TBS), and then incubated with anti-gastrin antibody. The slides were developed with nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP; BCIP/NBT substrate system for immuno-histochemistry and *in situ* hybridization, DakoCytomation) using the indirect immuno alkaline phosphatase method. By this double staining, gastrin-positive cells were stained dark blue and GLP-1-positive cells, red.

Statistical analysis

Data are expressed as mean ± SEM. Statistical significance was evaluated with the Mann–Whitney *U*-test at $P < 0.05$.

RESULTS

Phenotypes of glandular ducts in the fundic and pyloric areas of the stomach

We have judged the phenotypes of 1030 fundic and 2004 pyloric glandular ducts in areas of chronic gastritis using the gastric and intestinal mucous cell markers. The numbers of F, pseudo-P, GI-IM, I-IM-Pa(–), and I-IM-Pa(+) glands were found to be 214 (20.8%), 98 (9.5%), 265 (25.7%), 145 (14.1%), and 308 (29.9%), respectively, in the fundic areas. In the pyloric areas, the figures for P, GI-IM, I-IM-Pa(–), and I-IM-Pa(+) glands were observed to be 569 (28.4%), 1290 (64.4%), 40 (2.0%), and 105 (5.2%), respectively (Figs 1–3A–D,4). In the pyloric region, among IM glands, GI-IM occupied nearly 90%, whereas I-IM consisted of approximately 10%. In contrast, the ratio of I-IM was more than half in the fundic region compared to GI-IM ($P < 0.0001$).

Table 2 No. cells/gland and incidence (%) of each type of endocrine cell in the normal gastrointestinal tract (mean ± SEM)

	F	P	D	J	I	C
ChromograninA	3.93 ± 0.10	8.80 ± 0.47	5.79 ± 0.20	5.61 ± 0.14	2.88 ± 0.19	1.60 ± 0.05
	442/450	429/450	450/450	440/450	427/450	402/450
	98.22%	95.33%	100%	97.78%	94.89%	89.33%
Gastrin	0	6.96 ± 0.42	0.86 ± 0.09	0.01 ± 0.01	0	0
	0/450	446/450	230/450	3/450	0/450	0/450
	0%	99.11%	51.11%	0.67%	0%	0%
Somatostatin	0.32 ± 0.03	5.68 ± 0.47	0.67 ± 0.07	0.02 ± 0.01	0.08 ± 0.01	0.04 ± 0.01
	109/450	440/450	222/450	9/450	34/450	17/450
	24.22%	97.77%	49.33%	2.00%	7.56%	3.78%
Glicentin	0	0	0.04 ± 0.01	0.62 ± 0.05	1.73 ± 0.10	0.67 ± 0.04
	0/450	0/450	13/450	179/450	407/450	216/450
	0%	0%	2.89%	39.78%	90.44%	48.00%
GIP	0	0	1.06 ± 0.08	0.71 ± 0.04	0.55 ± 0.04	0
	0/450	0/450	281/450	208/450	201/450	0/450
	0%	0%	62.44%	46.22%	44.67%	0%
GLP-1	0	0	1.74 ± 0.09	0.68 ± 0.04	1.20 ± 0.06	0.24 ± 0.02
	0/450	0/450	355/450	208/450	344/450	94/450
	0%	0%	78.89%	46.22%	76.47%	20.09%

C, colonic glandular duct; D, duodenal glandular duct; F, fundic glandular duct; GIP, gastric inhibitory polypeptide; GLP-1, glucagon-like peptide-1; I, ileal glandular duct; J, jejunal glandular duct; P, pyloric glandular duct.

Figure 1 Normal pyloric glandular ducts. (A) HE staining. (B) Note human gastric mucin (HGM) in the cytoplasm in pyloric foveolar epithelial cells. (C) Paradoxical concanavalin A in the cytoplasm of pyloric glandular cells. (D) No staining of MUC2 in pyloric glandular ducts. (E) Chromogranin A detected in the endocrine cells of pyloric glands. (F) Gastrin is evident in endocrine cells of the pyloric glands. (G) No staining of gastric inhibitory polypeptide (GIP) is present in pyloric glands. Original magnification, 100.

Figure 2 The gastric and intestinal mixed phenotype of intestinal metaplasia (GI-IM). (A) HE staining. (B) Human gastric mucin (HGM) in the cytoplasm of GI-IM glandular ducts. (C) Paradoxical concanavalin A is partially present in the cytoplasm of GI-IM glandular epithelial cells. (D) MUC2 is present in the cytoplasm in GI-IM glandular ducts. (E) Chromogranin A in the endocrine cells of GI-IM glands. (F) Gastrin in the endocrine cells of the GI-IM (blue arrow). (G) Glicentin present in the endocrine cells in a case of GI-IM (red arrow). Note that gastrin-positive endocrine cells and glicentin-positive ones are in the same gland in serial sections. Original magnification, 100.

Expression of gastric and intestinal endocrine cell markers in normal gastrointestinal mucosa

Data for the distribution of endocrine cell markers in the normal gastrointestinal mucosa are summarized in Table 2. Gastrin-positive endocrine cells were predominantly detected in the normal pyloric and partially in the duodenal mucosa. Somatostatin-positive cells were also mainly detected in the normal pyloric, and partially in the fundic and duodenal mucosa. Glicentin-, GIP-, and GLP-1-positive endocrine cells were detected exclusively in the duodenum, small intestine and/or colon, but not in the normal gastric mucosa. Therefore as a consequence of these results, gastrin and somatostatin, although not completely specific, were chosen for gastric predominant endocrine cell markers, and glicentin, GIP, and GLP-1 to characterize the intestinal phenotype.

Expression of endocrine cell markers in glandular ducts in pyloric areas

The relative expression of gastric endocrine cell markers including gastrin and somatostatin statistically significantly decreased from P to GI-IM ($P < 0.0001$), and to I-IM ($P < 0.05$; Table 3). In contrast, expression of intestinal endocrine cell markers consisting of glicentin, GIP, and GLP-1 increased from P to GI-IM ($P < 0.0001$), but there were no significant differences between GI-IM and I-IM.

Expression of endocrine cell markers in glandular ducts in fundic areas

Gastrin-positive endocrine cells were not detected in normal F glands but were evident in pseudo-P glands, which significantly decreased to GI-IM ($P < 0.001$) (Table 4). No gastrin-positive cells were found in I-IM including both I-IM-Pa(–) ($P < 0.001$ vs pseudo-P) and I-IM-Pa(+) ($P < 0.05$ vs GI-IM) in fundic area, the latter being significantly lower than that in the pyloric mucosa ($P < 0.0001$; Table 4). Somatostatin, the other gastric endocrine cell marker, significantly decreased from pseudo-P to GI-IM and I-IM ($P < 0.05$). Conversely, the relative expression of the intestinal cell endocrine markers increased ($P < 0.001$), although again with slight decrease from GI-IM to I-IM.

Relation between mucous and endocrine cell markers in pyloric glandular ducts, GI-IM, and I-IM

In the normal P ducts, expression of gastric phenotypic mucous epithelial markers was observed, while an intestinal one was absent (Fig. 1A–D). Similarly, expression of gastric endocrine cell markers could be successfully visualized, but intestinal endocrine cell markers were seldom seen in the endocrine cells of pyloric glandular ducts (Fig. 1E–G). In the GI-IM glands, both gastric and intestinal endocrine markers were present in the endocrine cells, correlating with the phenotypic expression of the identical glandular cells (Fig. 2A–G). Regarding I-IM Pa(+) glands harboring only an intestinal mucous cell marker (Fig. 3A–D), endocrine cells demonstrated only intestinal endocrine peptides (Fig. 3E–G).

Glandular ducts with expression of both gastric and intestinal endocrine cell markers in the fundic and pyloric areas of stomach

The numbers of glands with gastric and intestinal mixed endocrine cells/total glands are summarized in Table 5. The

Table 3 No. cells/gland and incidence (%) of each type of endocrine cell in phenotypically classified glands in the pyloric area (mean ± SEM)

	P	GI-IM	I-IM-Pa(–)	I-IM-Pa(+)
Chromogranin A	6.35 ± 0.19	7.34 ± 0.16	5.58 ± 0.72	5.43 ± 0.31
	564/569	1276/1290	40/40	104/105
	99.12%	98.91%	100%	99.05%
Gastrin	5.32 ± 0.20	0.22 ± 0.02*	0.02 ± 0.02**	0.05 ± 0.02**
	510/569	158/1290	1/40	5/105
	89.63%	12.25%	2.50%	4.76%
Somatostatin	1.72 ± 0.07	0.48 ± 0.03*	0.13 ± 0.05**	0.12 ± 0.04**
	442/569	339/1290	5/40	11/105
	77.68%	26.28%	12.50%	11.43%
Glicentin	0.16 ± 0.06	3.31 ± 0.13*	1.34 ± 0.46*	1.49 ± 0.27*
	14/569	879/1290	12/40	63/105
	2.46%	68.14%	30.00%	60%
GIP	0.06 ± 0.02	1.27 ± 0.06*	1.29 ± 0.23*	1.52 ± 0.20*
	18/569	564/1290	22/40	67/105
	3.16%	43.72%	55.00%	63.81%
GLP-1	0.08 ± 0.03	3.00 ± 0.12*	1.68 ± 0.35*	2.18 ± 0.20*
	15/569	875/1290	23/40	83/105
	2.63%	67.83%	57.50%	79.05%

GI-IM, gastric and intestinal mixed phenotype IM; GIP, gastric inhibitory polypeptide; GLP-1, glucagon-like peptide-1; I-IM-Pa(–), solely intestinal phenotype IM without Paneth cell; I-IM-Pa(+), solely intestinal phenotype IM with Paneth cell; IM, intestinal metaplasia; P, pyloric glandular duct.
*$P < 0.0001$, compared with P. **$P < 0.05$, compared with GI-IM.

Table 4 No. cells/gland and incidence (%) of each type of endocrine cell in phenotypically classified glands in the fundic area (mean ± SEM)

	F	Pseudo-P	GI-IM	I-IM-Pa(–)	I-IM-Pa(+)
ChromograninA	2.29 ± 0.11	5.43 ± 0.20	6.13 ± 030	4.61 ± 0.31	4.17 ± 0.19
	205/214	96/98	264/265	145/145	306/308
	95.79%	97.96%	99.62%	100%	99.35%
Gastrin	0	1.03 ± 0.12	0.03 ± 0.02*	0*	0**,***
	0/214	31/98	5/265	0/145	0/308
	0%	31.63%	1.89%	0%	0%
Somatostatin	0.27 ± 0.06	1.20 ± 0.19	0.36 ± 0.05*	0.09 ± 0.04**	0.09 ± 0.02**
	56/214	26/98	57/265	8/145	21/308
	26.17%	26.53%	21.51%	5.52%	6.82%
Glicentin	0	0.01 ± 0.01	4.06 ± 0.32*	1.16 ± 0.36*	0.80 ± 0.10*
	0/214	1/98	192/265	31/145	101/308
	0%	1.02%	72.45%	21.38%	32.80%
GIP	0	0.01 ± 0.01	0.58 ± 0.07*	0.50 ± 0.07*	0.46 ± 0.05*
	0/214	1/98	88/265	47/145	83/308
	0%	1.02%	33.21%	32.41%	26.95%
GLP-1	0	0	3.08 ± 0.24*	1.39 ± 0.24*	1.03 ± 0.09*
	0/214	0/98	189/265	63/145	135/308
	0%	0%	71.32%	43.44%	43.83%

F, fundic glandular duct; GI-IM, gastric and intestinal mixed phenotype IM; GIP, gastric inhibitory polypeptide; GLP-1, glucagon-like peptide-1; I-IM-Pa(–), solely intestinal phenotype IM without Paneth cell; I-IM-Pa(+), solely intestinal phenotype IM with Paneth cell; IM, intestinal metaplasia; pseudo-P, pseudo pyloric glandular duct.

*$P < 0.001$, compared with pseudo-P. **$P < 0.05$, compared with GI-IM. ***$P < 0.0001$, compared with corresponding figure in Table 3.

incidence of glandular ducts with GI-mixed endocrine cells was demonstrated in 53 (5.1%) of 1030 glandular ducts in the fundic areas, and 347 (17.3%) of 2004 glandular ducts in the pyloric areas. In the fundic areas, the numbers and incidences of glandular ducts with GI-mixed endocrine cells in F, pseudo-P, GI-IM, I-IM-Pa(–) and I-IM-Pa(+) were demonstrated to be 1 (0.5%), 1 (1.0%), 44 (16.6%), 3 (2.1%) and 4 (1.3%), respectively. They were observed more frequently in GI-IM than in others ($P < 0.0001$). In the pyloric areas, the corresponding figures for P, GI-IM, I-IM-Pa(–) and I-IM-Pa(+) glandular ducts were observed to be 23 (4.0%), 306 (23.7%), 7 (17.5%) and 11 (10.5%), respectively. GI-mixed endocrine cells were also detected statistically more frequently in GI-IM than in P and in I-IM-Pa(+) glandular ducts. There was a significant difference between P and I-IM-Pa (+) with regard to the colocalization of gastric and intestinal endocrine cell markers.

Double staining of gastrin and GLP-1

Double immunostaining for gastrin and GLP-1 revealed existence of gastric and intestinal endocrine cells in the same gland featuring GI-mixed IM. We evaluated 300 glandular ducts and found 58 glands having both gastrin-positive cells and GLP-1-positive cells (19.3%). Of 58 glands, 11 glands (19.0%) harbored gastrin and GLP-1 double positive endocrine cells at the single-cell level (Fig. 5A,B).

DISCUSSION

Our present data provide clear evidence that the phenotypes of endocrine cells are strongly associated with those of mucous cells in IM. In particular, expression of both gastric and intestinal endocrine cell markers is observed more frequently in the endocrine cells of GI-IM glandular ducts than other phenotypes at the cellular level, as well as the glandular level. In C3H/HeN BALB/c chimeric mice, we have previously shown that each gland is derived from a single progenitor cell in a gastrointestinal tract, based on clonality analysis using a strain specific antibody.[30,31] Thus, we consider that all of the different types of mucous and endocrine cells not only in normal but also in intestinal metaplastic glands may be derived from a single progenitor cell.

GI-IM, characterized by the coexistence of gastric and intestinal mucous markers, are easily found in chronic gastritis. The same glands harbor both gastric and intestinal mucous cells.[29] Goblet cells usually emerge among foveolar cells and begin to possess MUC2 intestinal mucin but still retain the MUC5AC gastric one.[10] Present data show that the changes of phenotypes in endocrine cells as well as mucous cells occur with the alternation from the pyloric gland to the intestinal metaplastic gland. The relative expression of the intestinal endocrine cell markers increased from P or pseudo-P to GI-IM or I-IM. Ito *et al.* have previously shown that the glicentin-positive cells are detected in intestinal metaplastic and not gastric glands, but with marked decrease in completely intestinalized mucosa.[32] In addition to these previous reports, the present analysis is the first to demonstrate the presence of GI-mixed endocrine cells.

We earlier showed that Sox2 and Cdx1/2 are gastric and intestinal specific transcription factors, respectively.[10,29] In isolated pyloric and intestinal metaplastic glandular ducts, the phenotypes of mucous cells were found to be strongly associated with these specific transcription factors. In isolated GI-IM glandular ducts, Sox2 and Cdx1/2 were both observed, as well as gastric and intestinal mucous cell

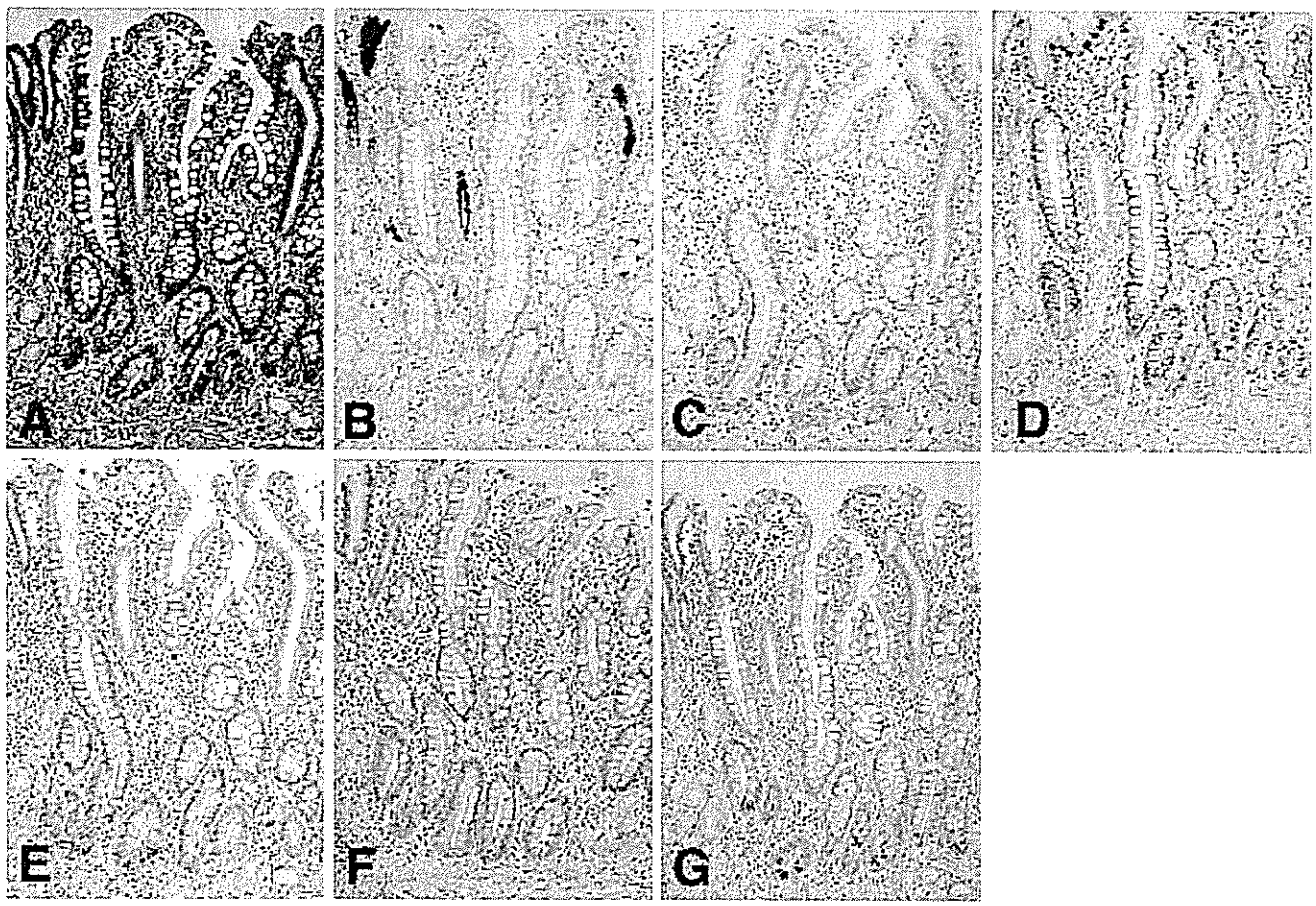

Figure 3 Solely intestinal phenotype of intestinal metaplasia (IM) with Paneth cells (I-IM-Pa(+)). (A) HE staining. (B) Note lack of human gastric mucin (HGM) in the cytoplasm of the I-IM-Pa(+) glands. (C) No staining of paradoxical concanavalin A in the cytoplasm of I-IM-Pa(+) glandular epithelial cells. (D) MUC2 is present in the cytoplasm of I-IM-Pa(+) glandular ducts. (E) Chromogranin A in the endocrine cells of I-IM-Pa(+) glandular ducts. (F) No staining of gastrin is evident in the endocrine cells of I-IM-Pa(+) glands. (G) Glucagon-like peptide-1 is present in the endocrine cells of I-IM-Pa(+) glands. Original magnification, 100.

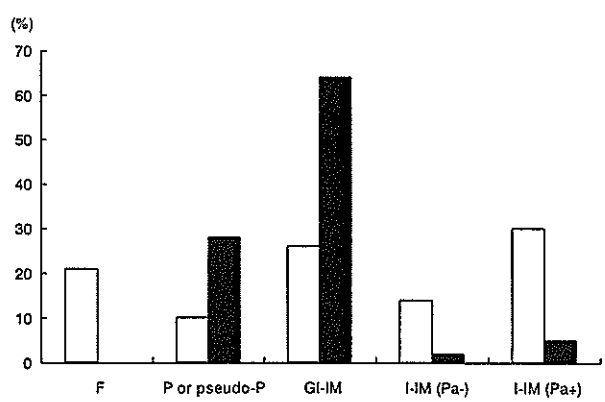

Figure 4 Incidence (%) of phenotypically classified glands in the (□) fundic and (■) pyloric regions. Distributions of GI-IM, I-IM-Pa(–), and I-IM-Pa(+) in these regions are significantly different ($P < 0.0001$). IM, intestinal metaplasia; F, fundic glandular duct; pseudo-P, pseudo pyloric glandular duct; P, pyloric glandular duct; GI-IM, gastric and intestinal mixed phenotype IM; I-IM-Pa(–), solely intestinal phenotype IM without Paneth cell; I-IM-Pa(+), solely intestinal phenotype IM with Paneth cell.

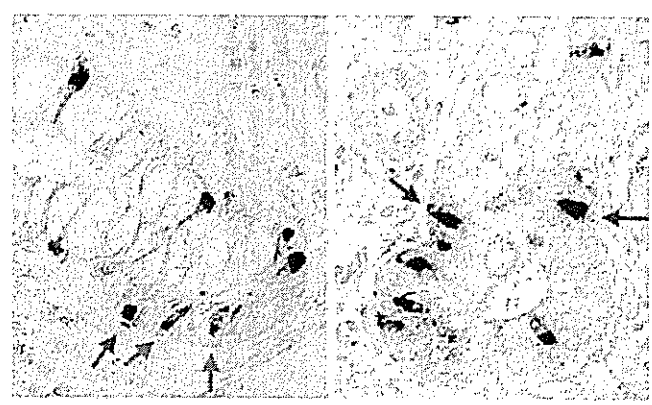

Figure 5 Two examples of double staining of gastrin and glucagon-like peptide (GLP)-1 in the gastric and intestinal mixed phenotype intestinal metaplasia (GI-IM). A mixture of gastrin- and GLP-1-positive endocrine cells are seen not only at the glandular level but also at the cellular level. Gastrin-positive cells (blue arrow), GLP-1-positive cells (red arrow), and endocrine cells positive for both (yellow arrow) are indicated. Original magnification, 200.

Table 5 No. glands with gastric and intestinal mixed endocrine cells/total glands

	F	Pseudo-P	P	GI-IM	I-IM-Pa(–)	I-IM-Pa(+)	Total
Fundic mucosa	1/214 (0.5%)*	1/98 (1.0%)*	–	44/265 (16.6%)	3/145 (2.1%)*	4/308 (1.3%)*	53/1030 (5.1%)
Pyloric mucosa	–	–	23/569 (4.0%)*	306/1290 (23.7%)	7/40 (17.5%)	11/105 (10.5%)**,***	347/2004 (17.3%)

F, fundic glandular duct; GI-IM, gastric and intestinal mixed phenotype IM; I-IM-Pa(–), solely intestinal phenotype IM without Paneth cell; I-IM-Pa(+), solely intestinal phenotype IM with Paneth cell; IM, intestinal metaplasia; P, pyloric glandular duct; pseudo-P, pseudo pyloric glandular duct.
*$P < 0.0001$, compared with GI-IM. **$P < 0.01$, compared with GI-IM. ***$P = 0.0012$, compared with P.

markers such as MUC5AC, MUC6, MUC2, and villin. Jenny *et al.* have previously demonstrated that neurogenin3 is required for endocrine cell fate specification in multipotent intestinal progenitor cells, whereas gastric endocrine development is both neurogrnin3 dependent and independent.[33] In addition, we consider that Cdx2 might be important in the regulation of the intestinal endocrine cell markers such as glicentin, GIP, and GLP-1, because its expression can be detected at the bottom of small intestinal and colonic glandular ducts. Recently, La Rosa *et al.* demonstrated that Cdx2 may be a sensitive and specific marker of midgut endocrine cells and endocrine tumors.[34] Thus specific transcription factors, including Cdx2, might play an important role in the intestinalization of both mucous and endocrine cells.

In pyloric glandular ducts with chronic gastritis, expression of the intestinal endocrine cell marker was low but present, in contrast to the normal pyloric mucosa without inflammation. With regard to the mucous cell markers, we earlier demonstrated the mRNA expression of *Cdx2* and *villin* in glandular ducts that are regarded as typical of G-type glands in the stomach of chronic gastritis.[29] Eda *et al.* have previously suggested that Cdx2 triggers the initiation and development of IM in chronic gastritis, from analysis of mRNA levels.[35] Satoh *et al.* described the expression of this in gastric epithelium of *H. pylori*-infected patients without IM.[36] Ishihara *et al.* proposed that *H. pylori* infection is a factor inducing glicentin in the gastric mucosa.[37] We therefore consider that change in transcriptional elements responsible for intestinal phenotypic expression of both mucous and endocrine cells might be the initial event in intestinalization of gastric mucosa in cases of chronic gastritis.

We have here demonstrated that the phenotype of endocrine cells in pseudopyloric glandular ducts is similar to those in the normal pyloric glandular ducts rather than those in the fundic glands. Furthermore, no gastrin-positive endocrine cells have been found in I-IM including both I-IM-Pa(–) and I-IM-Pa(+), resembling normal fundic glands. Thus, we consider the hypothesis that GI-IM in fundic mucosa could be preceded by pseudopyloric metaplasia with a similar pathogenesis occurring in pyloric glands, whereas some of the I-IM developing in fundic glands might be emerging directly from normal fundic glands without passing through pseudopyloric metaplasia. *Pancreatic–duodenal homeobox 1* (*Pdx1*) is a ParaHox gene, which contributes to genesis and development of pancreas, duodenum, and antrum. Pdx1 plays an important role in the development of pseudopyloric glands.[38] Thus, we consider that it is very important to clarify the regulation of mucous and endocrine cell markers from the viewpoint of transcriptional elements, discriminating pseudo-P glands from fundic mucosa in the human stomach. In the light of the clonal findings with C3H/HeN BALB/c chimeric mice, we consider that the alteration from fundic to pseudopyloric glands must be derived at the stem cell level.

In conclusion, our results suggest that the phenotypes of endocrine cells might be determined in tandem with those of their mucous counterparts in stomach glands. The alternation of phenotypes in mucous cells also involves those in endocrine cells and our results provide support for the concept that all of the different types of mucous and endocrine cells in normal and intestinal metaplastic glands might be derived from a single progenitor cell in each gland.

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RESEARCH COMMUNICATION

Suppressive Effects of Fruit-juice Concentrate of Prunus Mume Sieb. et Zucc. (Japanese apricot, Ume) on Helicobacter Pylori-induced Glandular Stomach Lesions in Mongolian Gerbils

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Abstract

Helicobacter pylori (*Hp*) infection is an important factor in human gastric disorders, including chronic active gastritis, peptic ulcers, intestinal metaplasia and cancer. Since epidemiologic studies overwhelmingly agree on a protective influence of fruits and vegetables in reducing the risk of gastric neoplasia and processed foods made from Prunus mume Sieb. et Zucc. (Japanese apricot or "Ume" in Japanese) are traditionally known for their miscellaneous medical effects, in the present study we investigated the efficacy of a fruit-juice concentrate of Japanese apricot (CJA) in the glandular stomach of *Hp*-infected Mongolian gerbils. *Hp*-inoculated gerbils were given CJA in their drinking water at concentrations of 1 and 3% for 10 weeks. The microscopic scores for gastritis and mucosal hyperplasia in the CJA groups were significantly lower than in the *Hp*-inoculated control group, with dose-dependence. Real-time PCR was performed to quantitate *Hp* by demonstrating urease A gene amount using gerbils' glyceraldehyde-3-phophate dehydrogenase (GAPDH) gene as an internal control. Average relative urease A gene dosage in the glandular stomach in the 1 and 3% CJA and *Hp*-inoculated control groups was 26.6 ± 11.6% (average ± SE), 30.3 ± 10.5%, 100 ± 40.9%, respectively, the fruit-juice concentrate causing significant lowering ($P<0.01$ and $P<0.05$, respectively, with 1 and 3%). These findings suggest that suppressive effects on gastric cancer development might also be expected as a result of decreased numbers of *Hp* and improvement of *Hp*-induced chronic active gastritis on administration of CJA.

Key Words: Helicobacter pylori - Mongolian gerbils - Prunus mume Sieb. et Zucc. (Japanese apricot, Ume) - glandular stomach - inflammation

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Introduction

Helicobacter pylori (*Hp*) is a major causative factor for gastric disorders and epidemiological evidence has accumulated indicating a significant relationship with chronic active gastritis, peptic ulcer, atrophic gastritis, intestinal metaplasia, and lymphoma or cancer development (Marshall and Warren, 1984; Nomura et al., 1991; Uemura et al., 2001). In 1994, the World Health Organization/ International Agency for Research on Cancer concluded that *Hp* is a 'definite carcinogen' based on the epidemiological findings (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 1994). However the pathogenic roles of *Hp* are still not fully understood. Eradication of *Hp* reduces the relapse rate of peptic ulcers and also results in histological resolution of chronic active gastritis (Hunt, 1996). The standard regimen for this purpose is adoption of triple therapy with a proton pump inhibitor in combination with two antibiotics, clarithromycin and amoxicillin (Misiewicz et al., 1997). Although the currently most effective treatment regimens cure about 90% of infections, 10% of patients remain *Hp* positive. Several factors contribute to treatment failure. These include patient compliance, bacterial resistance to antibiotics, and treatment related issues (Graham, 1998; Huang and Hunt, 1999). Therefore, it is important to find alternative approaches to

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control which are both effective and safe in terms of gastrointestinal protection from *Hp* associated diseases.

Epidemiologic studies overwhelmingly agree on the protective effect of fruits and vegetables in reducing the risk of gastric cancer (Serafini et al., 2002). In Japan, processed foods made from fruits of Prunus mume Sieb. et Zucc. (Japanese apricot or "Ume" in Japanese) are popular and traditionally considered to have miscellaneous medical benefit, such as antibacterial and fungicidal properties (Fujita et al., 2002; Maitani et al., 1985). Nomura et al. (Nomura et al., 1982) previously reported a significant negative association of ume (pickled plum) intake with intestinal metaplasia of the human stomach.

Mongolian gerbils can be easily infected with *Hp*, providing a good experimental animal to clarify the role of *Hp* in chronic active gastritis, peptic ulcers, intestinal metaplasia, and gastric cancer (Hirayama et al., 1996). We have established a gastric carcinogenesis model using these animals, and demonstrated that gastric cancer development is enhanced by *Hp* infection when they are treated with chemical carcinogens, like *N*-methyl-*N*-nitrosourea (MNU) or *N*-methyl-*N'*-nitrosoguanidine (MNNG) (Shimizu et al., 1999; Tatematsu et al., 1998). *Hp* eradication reduces the enhancing effect of *Hp* on gastric carcinogenesis (Cao et al., 2002; Nozaki et al., 2003).

In the present study, we therefore, investigated the efficacy of fruit-juice concentrate of Japanese apricot (CJA) in the glandular stomach of *Hp*-infected Mongolian gerbils.

Materials and Methods

Animals and Samples

A total of 60 specific pathogen-free male, four-week-old Mongolian gerbils (Meriones unguiculatus; MGS/Sea, Seac Yoshitomi, Ltd., Fukuoka, Japan) were housed in steel cages on hardwood-chip bedding in an air-conditioned biohazard room with a 12-h light/12-h dark cycle. They were given food (Oriental CRF-1, Oriental Yeast Co., Ltd., Tokyo, Japan) irradiated with 30 Gy γ-rays and autoclaved distilled water. The experimental design was approved by the Animal Care Committee of the Aichi Cancer Center Research Institute, and the animals were cared for in accordance with institutional guidelines. CJA was obtained from Minabegawa Village Office (Wakayama, Japan). CJA dissolved in distilled water at concentrations of 1 and 3% was freshly prepared three times per week for administration as drinking water.

Bacteria

Hp strain ATCC 43504 (American Type Culture Collection, Rockville, MD) was inoculated on Brucella agar plates (Becton Dickinson Co., Cockeysville, MD) containing 7% v/v heat-inactivated fetal bovine serum and incubated at 37°C under microaerobic conditions using an Anaero Pack Campylo (Mitsubishi Gas Chemical Co., Inc., Tokyo) at high humidity. Two days later, the bacteria grown on the plates were introduced into Brucella broth (Becton Dickinson Co.) supplemented with 7% v/v heat-inactivated fetal bovine serum and incubated under the same conditions for 24 h. The broth cultures of *Hp* were checked under a phase contrast microscope for bacterial shape and mobility. Samples containing about 1.0×10^8 colony-forming units per milliliter were used as the inoculum and delivered intra-gastrically (i.g.) using an oral catheter to gerbils fasted for 24 h. Uninfected gerbils underwent sham inoculation using the same sterile Brucella broth.

Experimental Protocol

The experimental design is illustrated in Fig. 1. Sixty gerbils were divided into 5 groups. *Hp* was inoculated into three of these groups at 1 experimental week. The other 2 groups received Brucella broth. CJA was administrated to *Hp*-inoculated and *Hp*-free animals in drinking water at the concentrations of 0, 1 or 3%, in all cases until the end of experiment at week 10. The gerbils were killed humanely at the end of the study period. All animals were subjected to deep ether anesthesia after 24 h fasting, laparotomized, and exsanguinated from the inferior vena cava, followed by excision of their stomachs. One half of each glandular stomach was fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) and routinely processed for histopathological examination, and the other half was quick frozen at -70°C for genomic DNA analysis.

Histopathological Analyses

Tissue sections were stained with hemotoxylin and eosin (H&E), Giemsa, and by immunohistochemistry for examination of *Hp* (anti-*Hp* serum, Dako Cytomation, Copenhagen, Denmark). The degree of chronic active gastritis was graded according to criteria modified from the Updated Sydney System (Dixon et al., 1996) by scoring the following parameters: mononuclear cell infiltration (0-3; 0, normal; 1, mild infiltration into lamina propria; 2, moderate infiltration into lamina propria; 3, marked infiltration into lamina propria and multiple lymphoid follicle formation); neutrophil infiltration (0-3; 0, none; 1, number of neutrophils in the pyloric mucosa in a line from the forestomach to the

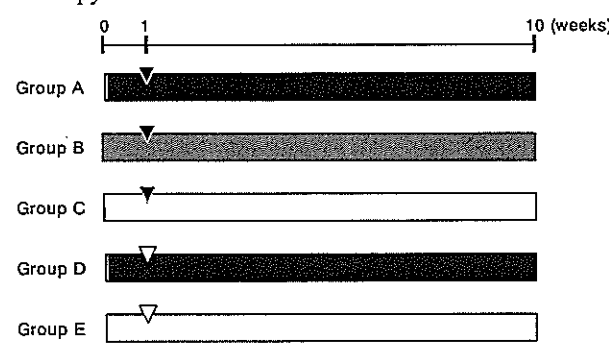

Figure 1. Experimental Design. Four week-old male Mongolian gerbils were used. Intragastric inoculation of Hp (closed triangles) or Brucella broth (open triangle). 3% (closed bar) or 1% (hatched bar) fruit-juice concentrate of Japanese apricot (CJA) was given in the drinking water. Control groups received unsupplemented water (open bar).

Table 1. PCR Primer Sequnces used in the Light Cycler Analysis

Description	Gene	Sequences	Product length (bp)	Accession No.
ua1	Urease A	5'-TGTTGGCGACAGACCGGTTCAAATC-3' (sense)	120	M60398
ua2		5'-GCTGTCCCGCTCGCAATGTCTAAGC-3' (antisense)		
ga1	GAPDH[a]	5'-AACGGCACAGTCAAGGCTGAGAACG-3' (sense)	118	AB040445
ga2		5'-CAACATACTCGGCACCGGCATCG-3' (antisense)		

[a] glyceraldehyde-3-phophate dehydrogenase

duodenum<50/mm; 2, 50-100/mm; 3, >100/mm); *Hp* density (0-3; 0, none; 1, mild *Hp* density; 2, moderate; 3, marked). The thickness of the pyloric mucosa was also measured at five randomly selected points in the foveolar epithelium.

Serology

Serum samples were used to measure the titer of anti-*Hp* IgG antibodies (GAP-IgG; Biomerica, Newport Beach, CA) by enzyme-linked immunosorbent assay (ELISA) using anti-gerbil IgG antibodies. The antibody titer was expressed by means of an arbitrary index (AI). A value greater than 1.37 AI was considered to be positive for *Hp* infection in both the infection and the control groups, as described earlier (Kumagai et al., 2001). Serum gastrin levels were measured using a gastrin radioimmunoassay kit (Gastrin-RIAkit II; Dainabot Co., Ltd., Tokyo).

Real-time Polymerase Chain Reaction and Relative Quantitative Analysis

Genomic DNA was extracted from glandular stomach tisue of gerbils using a DNeasy tissue kit (QIAGEN, Hilden, Germany). For *Hp* quantification, *Hp* specific urease A gene dosage within glandular stomachs of *Hp*-inoculated gerbils, relative quantitative real-time polymerase chain reaction (PCR) of Urease A was performed with the LightCycler system (Roche Diagnostics, Mannheim, Germany), using gerbil specific glyceraldehyde-3-phophate dehydrogenase (GAPDH) gene as an internal control. This was performed basically as described (Tsukamoto et al., 2001; Tsukamoto et al., 2004) using QuantiTect SYBR Green PCR (QIAGEN) with the optimal Mg^{2+} concentration at 2.5mM. The 5'-and 3'-primer sequences are listed in Table 1. Specificity of the PCR reaction was confirmed using the melting program provided with the LightCycler software. To further confirm that there was no obvious primer dimer formation or amplification of any extra bands, the samples were electrophoresed in 3% agarose gels and visualized with ethidium bromide after the LightCycler reaction. Relative quantitative analysis of *Hp* urease A gene expression was performed as earlier established using an internal control without the necessity of external standards (Tsukamoto et al., 2001; Tsukamoto et al., 2004), with values expressed as the percentage urease A gene expression, relative to the 100% in the *Hp*-inoculated control group (group C).

Statistics Analysis

The Mann-Whitney *U* test was applied to establish the significance of differences in urease A gene expression for corrected crossing points, microscopic score for gastritis, mucosal hyperplasia, titers of anti-*Hp* IgG antibodies, serum gastrin levels. *P* values <0.05 were considered to be statistically significant.

Results

Intake of CJA

Data for total intake of CJA per animal are shown in Table 2. CJA administration did not affect food intake or body weights.

Inflammation Score

Table 2 summarizes data for the efficacy of CJA in the glandular stomach of *Hp*-infected Mongolian gerbils. All animals of the *Hp*-inoculated control group (group C)

Table 2 Effects of Fruit-juice Concentrated of Japanese apricot (CJA) on Gastric Lesion of Mongolian Gerbils

Group	Administration	No. of gerbils	Microscopic score [SD]	Mucosal hyperplasia (mm) [SD]	Anti-*Hp* titer titer (AI) [SD]	Serum gastrin (pg/ml) [SD]	Total CJA intake (g/gerbil) [SD]
A	3 % CJA + Hp	20	3.00[a,b] [1.95]	0.34[c] [0.11]	4.01 [2.86]	101.13[d,e] [22.90]	10.54 [0.67]
B	1% CJA + Hp	21	4.38[a] [1.91]	0.42 [0.23]	5.89 [3.36]	133.19 [29.46]	4.76 [0.60]
C	Hp	10	8.00 [1.25]	0.50 [0.23]	6.47 [4.14]	150.31 [40.00]	0
D	3 % CJA	4	0	0.21 [0.02]	0.48 [0.17]	117.88 [18.54]	10.68 0
E	Control	5	0	0.23 [0.03]	0.18 [0.08]	140.88 [26.28]	0

[a] P<0.0001 vs. group C [b] P<0.05 vs. group B [c] P<0.05 vs. group C [d] P<0.005 vs. group C [e] P<0.001 vs. group B

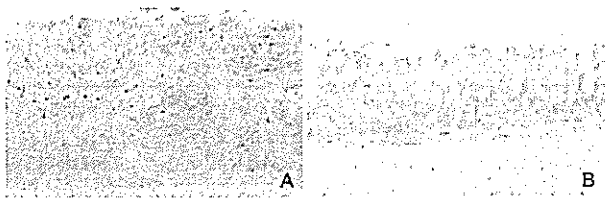

Figure 2. Histopathological Findings in the Pyloric Mucosa of Mongolian Gerbils Inoculated with *Hp*. (A) *Hp*-inoculated control group (group C). The glandular stomach shows hyperplastic change and severe infiltration of inflammatory cells (H&E, Original magnification, x50). (B) *Hp*-inoculated 3% CJA group (group A). The glandular stomach shows mild infiltration of inflammatory cells and mucosal hyperplasia (H&E, Original magnification, x50).

microscopically demonstrated severe gastritis with moderate to marked infiltration of inflammatory cells, mucosal hyperplasia with hemorrhagic erosion and moderate to marked *Hp* density mainly in the pyloric mucosa of glandular stomachs (Fig. 2A). The microscopic scores for the 1 and 3% CJA administrated group (groups A and B) were significantly lower than for the *Hp*-inoculated control group, with dose-dependence (Table 2). The thickness of the pyloric mucosa was also reduced dose-dependently in CJA administrated group, reaching significance in the 3% CJA group (Fig. 2B). No evidence of gastritis and mucosal hyperplasia was found in any *Hp*-free animals.

Antibody Titer and Serum Gastrin Level

Titer of anti-*Hp* antibodies in all *Hp*-inoculated groups were greater than the cut off values expect in one animal in group A, which was excluded from the analysis. There were no significant differences in antibody titers among groups A-C (Table 2). The values for serum gastrin were reduced dose-dependently in the CJA groups, and significantly with the 3% dose (group A) (Table 2).

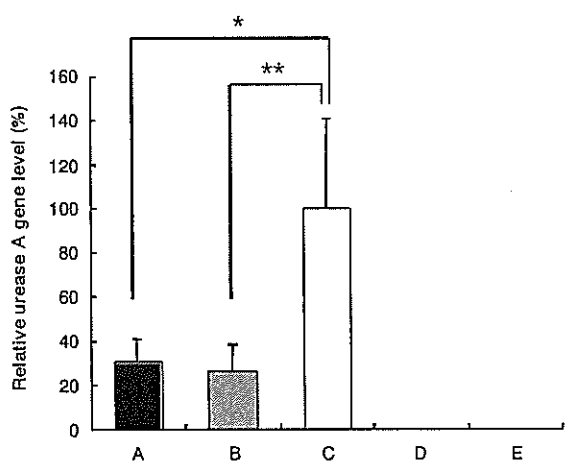

Figure 3. Relative Expression Levels of the Urease A Gene in Glandular Stomachs of Mongolian Gerbils. Values were set at 100% in group C and expressed as mean ± SE. Note decrease in relative urease A gene levels in groups A and B as compared to group C. *P<0.05 and **P<0.01, by the Mann-Whitney *U* test.

Quantification of Hp

Real-time PCR was performed to demonstrate expression of the urease A gene of *Hp*-inoculated groups using GAPDH as an internal control. Average relative urease A gene levels of glandular stomach in 1 and 3% CJA and *Hp*-inoculated control groups were 26.6±11.6% (average ±SE), 30.3±10.5% and 100±40.9%, respectively. The lowering by CJA was significant (P<0.01 and P<0.05, respectively, of 1 and 3%) (Fig. 3). Furthermore, no amplification of the urease A gene was detected in 4 of 20 animals (20%) in group A and 1 of 21 animals (4.8%) in group B, in addition to all the *Hp*-free animals.

Discussion

Our present data provide clear evidence that a fruit-juice concentrate of Japanese plums administered in the drinking water can suppress chronic active gastritis in the glandular stomachs of *Hp*-infected Mongolian gerbils in a dose-dependent manner, reducing urease A gene amount in the *Hp*-inoculated glandular stomach. In the 20% of 3% CJA and 4.8% of 1% CJA administered gerbils without detectable urease A gene, histological examination for *Hp* also proved negative, indicating the possibility that *Hp* had been eradicated in these animals. Rokbi et al. have previously demonstrated that real-time PCR is a powerful tool for the detection and quantification of *Hp* gene expression in the gastric mucosa (Rokbi et al., 2001) and PCR amplification of the *Hp* urease A gene is a highly sensitive and specific method for the diagnosis of *Hp* infection (Clayton et al., 1992).

The Japanese plum (ume), Prunus mume Sieb. et Zucc. (Rosaceae), has been traditionally used as a medical food in Japan and in Chinese traditional medicine, various parts of the plant are used. Although a number of reports have been published with concrete evidence that Japanese apricots are effective against diseases (Maitani et al., 1985), information on the mechanisms, for example of its antibacterial and fungicidal properties, is limited. It has been postulated that antioxidants may reduced cancer risk by modulating red-ox status, by preventing biologic oxidant, and by inhibiting the formation of carcinogen (Serafini et al., 2002). Utsunomiya et al. previously reported that fruit-juice concentrate of Japanese plum possesses a potent antioxidant activity (Utsunomiya et al., 2002). Iimuro et al. have shown that antioxidative effects of garlic may have suppressive effects on *Hp*-induced gastritis in Mongolian gerbils (Iimuro et al., 2002). We therefore hypothesize that antioxidative effects of CJA may have contributed to the suppression of chronic active gastritis in glandular stomach of *Hp*-infected Mongolian gerbils.

In addition, CJA harbors strong acids, including citric and malic acid (Chuda et al., 1999; Fujita et al., 2002), which may exert antibacterial action and cause environmental change in the stomach. Suppressive effects on gastric cancer development would be expected as a result of the decrease of quantity of *Hp* and improvement of *Hp*-induced chronic

active gastritis by administration of CJA. Actual ingredients which might be effective for *Hp*-induced chronic active gastritis have not been clarified but warrant further examination. Studies are now in progress to clarify the suppressive effect of gastric cancer development in gastric carcinogenesis model using Mongolian gerbils.

In conclusion, in this present study, we found CJA to suppress chronic active gastritis in the glandular stomachs of *Hp*-infected Mongolian gerbils. Therefore, CJA may have potential as a safe and inexpensive agent to control *Hp*-associated gastric disorders in Japan, including gastric neoplasia.

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ORIGINAL ARTICLE

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Expression of small intestinal and colonic phenotypes in complete intestinal metaplasia of the human stomach

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Abstract The incomplete intestinal metaplasia (IM) that is reported to be a risk factor for gastric carcinogenesis in man usually features sulfomucin production and thus is considered of colonic type. To cast light on the underlying mechanisms, we here examined the proportions of colonic and small intestinal phenotypes in IM by immunohistochemistry and real-time reverse transcription–polymerase chain reaction at the single isolated gland level. Carbonic anhydrase 1 (CA1) is a specific marker of colonic epithelial cells, whereas sucrase is specific to absorptive cells of the small intestine. Totals of 139 (23.5%) and 452 (76.5%) IM glands were judged to be CA1 positive and CA1 negative, respectively, in resected pyloric mucosa from cancer patients. The average score for MUC5AC in CA1-positive IMs was significantly lower than in CA1-negative counterpart tissue ($P<0.0001$), whereas the opposite was the case for sucrase ($P<0.0001$). High iron diamine–Alcian blue staining revealed CA1 expression to coincide with type I complete IM. The expression of CA1 mRNA strongly correlated with that of sucrase–isomaltase, and inversely with that of MUC5AC in isolated IM glands. In conclusion, CA1 could be colocalized with small intestinal proteins such as sucrase, but only rarely with the gastric mucin, MUC5AC. Its expression warrants further study, with the focus on stimulation and/or suppression mechanisms by gastric and intestinal transcription factors.

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Introduction

Intestinal metaplasia (IM) has been extensively studied as a possible premalignant condition in the human stomach [2, 24, 36, 52, 55]. However, many questions remain regarding its pathogenesis as well as the actual relationship to gastric cancers. Several classifications of IM have been suggested by pathologists. Kawachi et al. [16] first proposed division into complete and incomplete types on the basis of morphology. Jass and Filipe [14] described three grades of IM with classical mucin staining. These classifications are generally accepted, but are only based on intestinal properties and do not take into account the gastric properties that are still preserved in association [45]. We have therefore previously proposed a new IM classification based upon the cell differentiation status using both gastric and intestinal epithelial cell markers, such as MUC5AC, MUC6, MUC2, and villin [13, 45, 49]. Division is into two major types: a gastric-and-intestinal-mixed type (GI-IM) and a solely intestinal type (I-IM). Thus, it is important to analyze not only intestinal phenotypic expression but also the contribution of gastric elements within IM glands.

Several studies have provided evidence that type III intestinal metaplasia (incomplete sulphomucin-secreting intestinal metaplasia) may be a risk factor for gastric carcinogenesis [10, 12], although this remains controversial [6]. Classification based upon mucin secretion patterns as well as morphology has allowed division into a "small intestine type" and a "colonic type" [32, 46], although this has hitherto not been confirmed with small intestinal and colonic specific markers. With regard to the distribution of intestinal epithelial cell markers such as villin and MUC2 in the normal human alimentary tract, most are present in both the colon and the small intestine. However, carbonic anhydrase 1 (CA1) is found only in epithelial cells located on the luminal side of colonic mucosa, and not in the small

intestine [29, 35]. Conversely, sucrase is detectable on the luminal surfaces of mature small intestinal absorptive cells, but only rarely in colon [51]. Therefore, regarding intestinal phenotypic expression, CA1 can be used to indicate the colonic phenotype, whereas sucrase is representative of a small intestinal phenotype. The clinicopathologic significance of colonic and small intestinal phenotypes has yet to be clarified in detail for IM. Indeed, to our knowledge, there have been no reports of expression of CA1 in IM.

Most studies for gene expression for IM have been performed using whole tissue [7, 19]. However, it is impossible to avoid contamination of epithelial cell elements with stromal or inflammatory cells using this approach. We have previously established a novel "gland isolation technique" for the isolation of pyloric and IM glands and performance of relative quantitative real-time reverse transcription–polymerase chain reaction (RT-PCR) analysis of gastric and intestinal transcription factors as well as differentiation marker genes [49]. This technique made it possible to analyze gene expression separately in each isolated gland without contamination [20]. In isolated GI-IM glands, we confirmed the expression of both gastric and intestinal transcription factors as well as differentiation markers [49].

In the present study, we evaluated the transcription of CA1, sucrase–isomaltase, and MUC5AC by relative quantitative real-time RT-PCR in individual isolated glands and analyzed the interrelationships among the markers. We also examined the expression of these markers in IMs by immunohistochemistry, with reference to histological and phenotypic classification using other epithelial cell markers.

Materials and methods

Human tissue

A total of 15 stomachs, resected because of gastric cancer at Aichi Cancer Center Hospital, were investigated. This study using human tissue was conducted with approval from the Ethical Review Board of Aichi Cancer Center and after obtaining informed consent from the patients. Non-neoplastic pyloric mucosa distant from carcinomas, at least 10 cm from the tumor margins, was collected and used for RNA analysis after gland isolation as previously described [49]. After surgery, samples were fixed in 10% buffered formalin and embedded in paraffin. Serial sections were cut at 4 μm, one being stained with hematoxylin and eosin (H&E) for routine histological and others for immunohistochemical assessments as previously described [23]. Fresh normal mucosal samples of gastrointestinal tract (antrum of the stomach, jejunum, sigmoid colon) and specimens from each IM region in the pyloric mucosa with chronic gastritis were also obtained with informed consent from other patients and stored at −80°C as previously described [19].

Mucin histochemistry and immunohistochemistry

For mucin histochemistry, Alcian blue (AB) (pH 2.5) staining was performed as described [49]. Immunohistochemical staining was carried out with antibodies against the following antigens: CA1 (Chemicon International, Temecula, CA, USA), Cdx2 (BioGenex, CA, USA), MUC5AC (Novocastra Laboratories, Newcastle upon Tyne, UK), MUC2 (Novocastra), and villin (BD Transduction Laboratories, Lexington, KY, USA). The polyclonal rabbit anti-sucrase antibody was kindly provided by Dr. Kazuyuki Hirano, Department of Pharmaceutics, Gifu Pharmaceutical University, Gifu, Japan. The precise procedures for immunohistochemical techniques were as previously described [21–23, 49–51]. Two independent pathologists (T. T. and K. I.) judged the histology, mucin histochemistry, and immunohistochemical staining of epithelial cell markers, including Cdx2. Reactivity for CA1, MUC5AC, and sucrase was scored according to the rate of mucous cells above the proliferative cell zone in each gland of the pyloric areas on a 3-point scale: score 0, no positive mucous cells; score 1, 0%<positive cells≤35%; score 2, 35%<positive cells≤70%; score 3, 70%<positive cells. Results for CA1 or MUC5AC were considered positive with a score 1 or more. Using the scoring criteria, we evaluated 850 glands, 259 pyloric and 591 IM, in the pyloric region of 10 CA1-positive surgically resected IM cases. Similarly, 444 IM glands were judged for high iron diamine (HID)-AB staining patterns and classified into three subtypes as detailed by Jass and Filipe [14]. The data were analyzed by the Mann–Whitney's U test or the Fisher's exact test for differences between the groups. P values <0.05 were considered statistically significant.

Double immunohistochemical staining of CA1 and sucrase

Double staining of CA1 and sucrase was achieved basically as we previously described [28, 44]. Sections were deparaffinized, hydrated with ethanol, and autoclaved at 121°C in water for 10 min. They were then incubated with goat polyclonal anti-CA1 antibodies at 4°C overnight. Sections were then exposed to biotin-labeled anti-goat IgG and avidin–biotin peroxidase complex (Vector), and binding sites were visualized with DAB as the chromogen. Then, the antibody complex was removed with autoclaving, and the slides were thoroughly washed with Tris-buffered saline (TBS), then incubated with rabbit polyclonal anti-sucrase antibody at 4°C overnight, followed by alkaline phosphatase-labeled chicken anti-rabbit IgG (Chemicon). Color was developed using the DAKO fast red substrate system (DakoCytomation, Glostrup, Denmark) in the presence of levamisole (Sigma Chemical Co., St. Louis, MO, USA), and sections were counter-stained with hematoxylin. By the double staining, CA1-positive cells were stained brown, and sucrase-positive cells red.

Gland isolation

Gland isolation was performed as previously described [20, 49]. Remaining portions of resected epithelium were injected with calcium- and magnesium-free Hanks' balanced salt solution containing 30 mM ethylenediaminete traacetic acid (EDTA-HBSS) submucosally, incubated in EDTA-HBSS, and shaken for 15 min at 37°C. Then the mucosa was scraped off with a scalpel. Isolated glands were washed in phosphate buffered saline, fixed in 70% ethanol for a few hours, dehydrated with 95% ethanol, and stored at −20°C until use.

Mucin cytochemistry and classification of isolated glands

The phenotypic classification of isolated glands was performed with AB staining as described earlier, based on the presence of goblet cells and pyloric glands, instead of with detailed immunohistochemical analyses to avoid degradation of RNA [45, 49]. Glands exhibiting AB-positive goblet cells in the upper part and an AB-negative bottom region (pyloric gland) were judged as GI-IM. Examples with many AB-positive goblet cells throughout and loss of AB-negative pyloric glands were classified as I-IM. Typical glands as shown in Fig. 6 were used in this study, and transient types were discarded to avoid confusion. This simple method for the classification of isolated glands were found to fit well with histological and immunohistochemical analyses [49].

Total RNA isolation from normal gastrointestinal whole tissues and isolated glands

Total RNAs from frozen tissues were extracted with TRIzol total RNA isolation reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions [19, 22, 47]. For the stomach, neighboring tissues were evaluated for their histology either of the presence or absence of IM irrespective of the presence of gastric component. Individual ethanol-fixed isolated classified glands were transferred to 0.5-ml microfuge tubes under an inverted microscope and subjected to total RNA isolation as described [48, 49]. A total of 12 isolated classified glands were analyzed separately.

Relative quantitative analysis for isolated glands

First strand cDNAs were synthesized as previously reported [48, 49] using the Thermoscript RT-PCR System (Invitrogen) according to the manufacturer's instructions. Relative quantitative PCR for CA1, sucrase–isomaltase, and MUC5AC was performed with the LightCycler system (Roche Diagnostics, Mannheim, Germany), using β-actin as an internal control [49]. The 5′ and 3′ primer sequences are listed in Table 1. PCR was performed basically as described [48, 49] using a SYBR Green PCR Core Reagents kit (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). Quantification was performed as earlier established using β-actin as an internal control. The values for corrected crossing points were calculated by subtraction of the crossing points of the gene of interest from that of β-actin, then used for statistical analyses applying the Mann–Whitney *U* test as earlier described [48, 49]. Specificity of the PCR reaction was confirmed using the melting program provided with the LightCycler software. To further confirm that there was no obvious primer dimer formation or amplification of any extra bands, samples were electrophoresed in 2.5% agarose gels and visualized with ethidium bromide after the LightCycler reaction. Total RNA samples without RT provided a control for PCR amplification (data not shown). The sample correlation coefficient was used for comparisons. A correlation between two factors with relative quantitative real-time RT-PCR was evaluated using Spearman's nonparametric correlation coefficient by rank. P values <0.05 were considered statistically significant.

Results

The expression of CA1, sucrase, and MUC5AC in normal gastrointestinal mucosa, and in pyloric mucosa having IM glands, associated with chronic gastritis.

Figure 1 shows double staining of CA1 and sucrase in normal colon (Fig. 1a), jejunum (Fig. 1b), and pyloric mucosa (Fig. 1c). Expression of CA1 was detected in cy-

Table 1 Primer sequences for real-time RT-PCR

Target genes	Orientation	Sequence	Product length (bp)	GeneBank accession nos.
Carbonic anhydrase 1	Upper	TGACCCCTCTACTCTCCTTCCT	187	NM_001738
	Lower	CATGGGGACAGCGTTATCAC		
Sucrase–isomaltase	Upper	CAAATATCGACTCGCCTGCCATCAG	193	XM_010952
	Lower	CACCATGAGCATTGCCCTCCTCTT		
MUC5AC	Upper	CAGAAATCCAGGACAACCAC	192	AJ298317
	Lower	AACAGGGCTCGGAGTAGTTTTA		
Beta-actin	Upper	TGGCACCCAGCACAATGAAG	142	M10277
	Lower	GATGGAGGGGCCGGACTC		

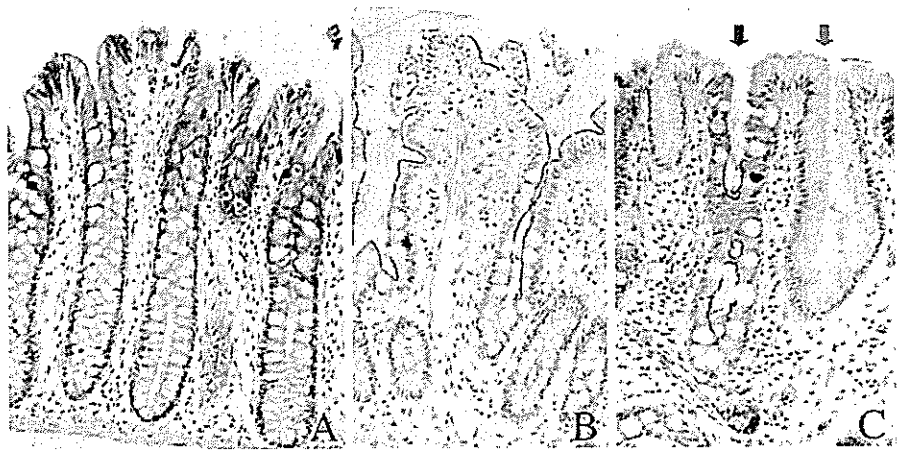

Fig. 1 Double staining of CA1 and sucrase in normal colon, jejunum, and stomach with IM. a Expression of CA1 is clearly evident in epithelial cells located on the luminal side of colonic mucosa, but not in the jejunum. b Sucrase, on the other hand, is present on the luminal surfaces of mature small intestinal absorptive cells, but not in the colon. c In the pyloric region with chronic gastritis, gastric foveolar cells of pyloric glands have neither CA1 nor sucrase expression (*red arrow*), whereas IM glands demonstrate both CA1 cytoplasmic staining and sucrase expression at their luminal surfaces (*blue arrow*). Original magnification, ×200. *CA1*, carbonic anhydrase 1; *IM*, intestinal metaplasia

toplasm of colon epithelial cells, especially on the luminal side of colonic mucosa, but not in the jejunum. Sucrase, on the other hand, was present on the luminal surfaces of mature small intestinal absorptive cells, but not in the colon. Neither CA1 nor sucrase expression was detected in the gastric foveolar cells of pyloric glands (Fig. 1c, red arrow). However, in the pyloric region with chronic gastritis, some IM glands had both CA1 cytoplasmic staining and sucrase expression at their luminal surfaces (Fig. 1c, blue arrow). Erythrocytes in blood vessels beneath the glands cross-reacted with the anti-CA1 antibody (Fig. 1c). Figure 2 shows results of RT-PCR analyses of CA1, sucrase–isomaltase, and MUC5AC in the normal alimentary tract including the colon, jejunum, and antrum of the stomach, with or without IM. Products of β-actin detected as a 142-bp band were used as an internal and sample quality control. CA1 RT-PCR products were detected as a 187-bp band in the colon, but not the antrum or jejunum, whereas RT-PCR products of sucrase–isomaltase were detected as a 193-bp band in the jejunum, but not the antrum or colon, compatible with the immunohistochemistry results. MUC5AC RT-PCR products were detected as a 192-bp band in the normal antrum of stomach, but not in the colon or jejunum. In pyloric mucosa having IM glands, PCR products of all of the abovementioned epithelial cell markers proved detectable (Fig. 2).

The expression of MUC5AC and sucrase in both CA1-positive and CA1-negative IM glands

Next, we evaluated the expression of gastrointestinal differentiation markers in IM at the gland level in serial sections. Figure 3 illustrates immunoreactivitiy of MUC5AC, CA1, sucrase, MUC2, villin, and Cdx2 in IM glands. Elevated MUC5AC expression was observed in CA1-negative mucous cells of GI-IM glands (Fig. 3b and c, red arrow), compared with the CA1-positive I-IM (Fig. 3b and c, blue arrow). The expression of sucrase was more strongly detected on the luminal surfaces of CA1-positive IM gland cells than in CA1-negative IM glands (Fig. 3d). MUC2, villin, and Cdx2 expression was observed in intestinal metaplastic cells irrespective of CA1 expression (Fig. 3e–g, respectively).

To quantitatively clarify the relation of CA1, MUC5AC, and sucrase, 850 glands, comprising 259 pyloric and 591 IM glands, were immunohistochemically evaluated in the pyloric regions of 10 CA1 positive surgically resected cases among a total of 15 cases. In the pyloric glands, all mucous cells demonstrated MUC5AC staining, whereas neither CA1 nor sucrase-positive cells were observed. Among 591 IM glands, totals of 139 (23.5%) and 452 (76.5%) were judged to be CA1 positive and CA1 negative, respectively. The average score for MUC5AC in CA1-positive IM was lower than that in CA1-negative IM with statistical significance ($P<0.0001$) (Fig. 4a). On the other

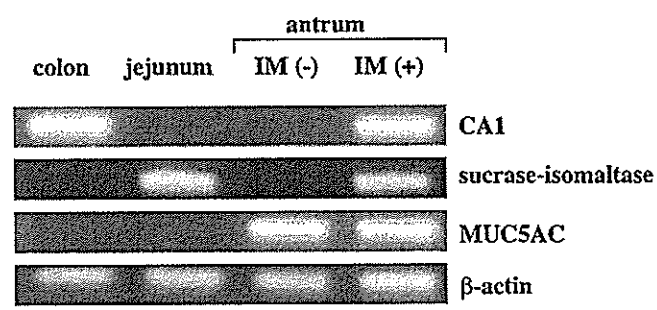

Fig. 2 CA1, sucrase–isomaltase, MUC5AC, and β-actin mRNA expression in colon, jejunum, and antrum of the stomach, with or without IM. CA1 is expressed only in colon, and sucrase–isomaltase only in jejunum. Antral mucosa possesses MUC5AC mRNA and ectopically expresses CA1 and sucrase–isomaltase with occurrence of IM. Products of β-actin detected as a 142-bp band were used as an internal control. *CA1*, carbonic anhydrase 1; *IM*, intestinal metaplasia

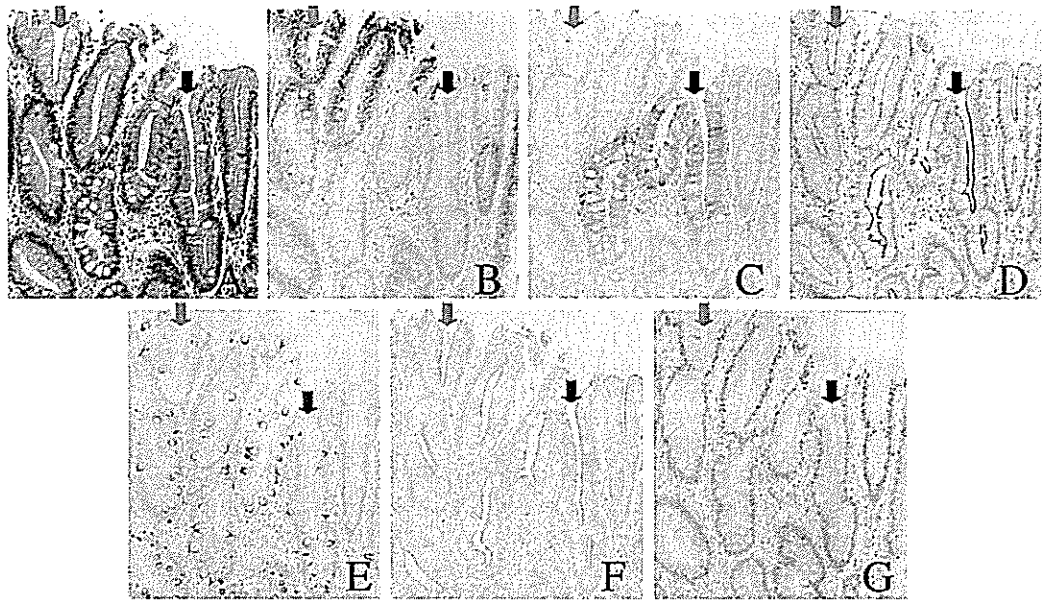

Fig. 3 Histology of IM and expression of gastric and intestinal markers. **a** H&E staining; **b** MUC5AC is apparent in the cytoplasm of CA1-negative (*red arrow*) IM glands, with little expression detected in CA1-positive (*blue arrow*) IM glands; **c** CA1 cytoplasmic staining is observed in the mucous cells of some IM glands (*blue arrow*), but not in others (*red arrow*); **d** Sucrase is apparent at the luminal surfaces of IM glands; **e** MUC2 is detectable in the cytoplasm of IM glands; **f** Villin is positive at the luminal surfaces of IM glands; **g** Cdx2 nuclear staining is apparent in IM glands. Original magnification, ×200. *CA1*, carbonic anhydrase 1; *IM*, intestinal metaplasia

hand, the average score for sucrase in CA1-positive IM was significantly higher than that in CA1-negative tissue (P<0.0001) (Fig. 4b). Considering sucrase expression, many of IM glands (80.4%=475/591) possessed sucrase on the luminal surface. Sucrase-negative IM glands in turn had high level of MUC5AC, 115 IM glands showing score 3 of MUC5AC and only 1 gland having score 2. Thus, sucrase preferred to express more strongly in I-IM than in GI-IM.

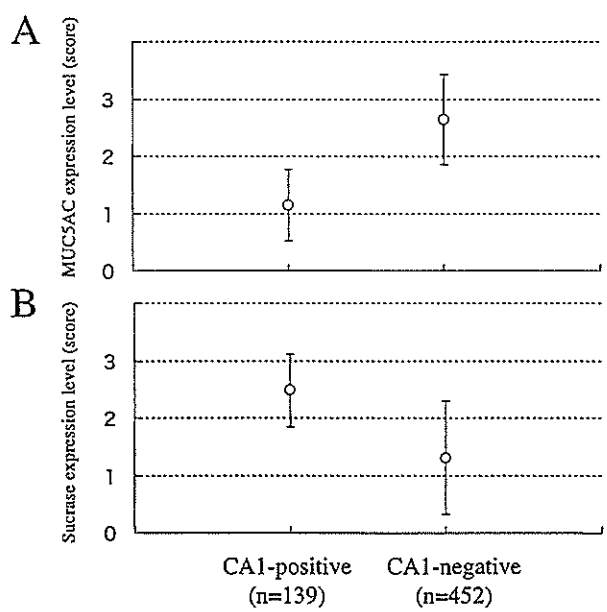

Fig. 4 Relations among MUC5AC, sucrase, and CA1 expression in IM glands by immunohistochemistry. **a** The scores for MUC5AC expression in CA1-positive and CA1-negative IMs are 1.15±0.63 and 2.64±0.78 (average±SD) (P<0.0001). **b** The scores for sucrase expression in CA1-positive and CA1-negative IMs are 2.48±0.63 and 1.31±0.98 (P<0.0001). *CA1*, carbonic anhydrase 1; *IM*, intestinal metaplasia

Comparison of HID-AB staining based IM subtypes with CA1/MUC5AC expression

Inasmuch as HID-AB staining has been widely used for classification of IM in terms of small intestinal sialomucin and colonic sulfomucin expression, we compared the localization of these mucins and the presence of CA1 and MUC5AC proteins. A total of 444 IM glands were evaluated for mucin expression and were classified into 256 type I glands having no mucins in columnar cells but with goblet cells, 104 type II glands with blue-stained columnar cells possessing sialomucins, and 84 type III glands with brown-stained columnar cells producing sulphomucins. Then, serial sections were assessed for expression of a colonic marker, CA1, and also a gastric marker, MUC5AC, for each subtype (Fig. 5 and Table 2). The number of glands with CA1 expression proved higher in type I complete IM compared to types II and III incomplete IM (P<0.005). Furthermore, there turned out to be no differences between the types II and III in terms of CA1 expression, and no correlation with colonic sulfomucin expression was apparent. Considering the gastric marker, type I glands possess less MUC5AC core protein than types II and III (P<0.0001), the last demonstrating the most MUC5AC-positive glands (79.8 and 90.5%, respectively), although this was not statistically significant (P=0.066).

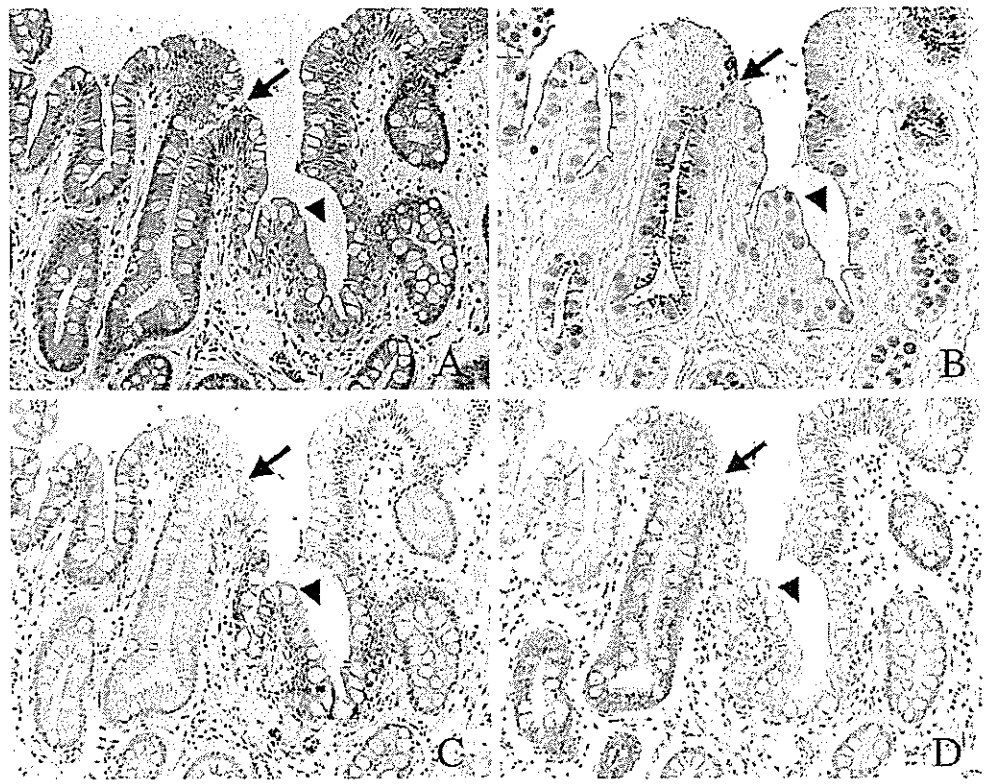

Fig. 5 HID-AB staining patterns and expression of CA1 and MUC5AC in IM glands. a H&E staining; b HID-AB staining showing subtype III (*arrow*) and subtype I (*arrowhead*) IM glands; c CA1 cytoplasmic staining in the columnar cells in one type I IM gland (*arrowhead*), but not in another (*arrow*); d MUC5AC is apparent in the cytoplasm of type III gland (*arrow*) IM glands, but expression is limited in type I (*arrowhead*) IM glands. Original magnification, ×125

Relative mRNA expression of CA1, sucrase–isomaltase, and MUC5AC in isolated GI-IM and I-IM glands by real-time RT-PCR

To determine the reliability of the data from the immunohistochemical analyses, we evaluated the relative mRNA levels for CA1, sucrase–isomaltase, and MUC5AC by real-time PCR in the isolated IM glands. Twelve isolated IM glands were stained with AB and phenotypically divided into seven GI-IMs and five I-IMs, as shown in Fig. 6. RNA extraction was performed separately for each gland, and real-time RT-PCR analysis of CA1, sucrase–isomaltase, and MUC5AC was carried out at the single gland level. Average (±SE) normalized cycle numbers for MUC5AC, sucrase–isomaltase, and CA1 in GI-IM and I-IM are described in Table 3. The level of MUC5AC mRNA was drastically lower in I-IM compared to GI-IM ($P<0.005$). Inversely, expression of sucrase–isomaltase and CA1 mRNAs was increased from GI-IM to I-IM ($P<0.05$ and $P<0.005$, respectively). Figure 7a shows the relation between CA1 and MUC5AC in the 12 isolated IM glands. The relative MUC5AC mRNA expression was inversely correlated with that of CA1 ($P<0.05$). Figure 7b shows the positive link between CA1 and sucrase–isomaltase in the isolated IM glands ($P<0.05$).

Discussion

The present data provide clear evidence that CA1 expression correlates with sucrase expression in IM glands of pyloric mucosa, and inversely with MUC5AC expression, as assessed by immunohistochemistry and relative quantitative real-time RT-PCR. Some MUC5AC expression was observed in the cytoplasm of CA1-negative mucous cells in areas of IM, and the data from real-time RT-PCR at the single isolated gland level support the results of immunohistochemical analysis. We have previously suggested that intestinalization of gastric mucosa may represent a kind of homeotic transformation [45, 49]. Cdx2, an intestinal homeobox gene, is an important factor for the development of IM [7, 19], its gastric expression alone being sufficient to induce intestinal metaplasia in mice [25, 33]. Cdx2 mRNAs are widely present in the human intestinal

Table 2 Correlation of HID-AB staining and expression of CA1 and MUC5AC

	HID-AB		
	Type I	Type II	Type III
No. of glands (%)	256	104	84
CA1 (+)	35 (13.7)*	3 (2.9)	2 (2.4)
(−)	221 (86.3)	101 (97.1)	82 (97.6)
MUC5AC (+)	97 (37.9)**	83 (79.8)	76 (90.5)
(−)	159 (62.1)	21 (20.2)	8 (9.5)

*$P<0.005$ vs. type II, $P<0.005$ vs. type III
**$P<0.0001$ vs. type II, $P<0.0001$ vs. type III

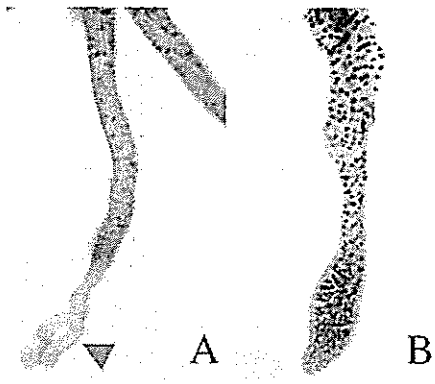

Fig. 6 Isolated IM glands from pyloric mucosa. a Isolated GI-IM gland with the presence of pyloric cells (*arrowhead*); b isolated I-IM gland with more Alcian blue-positive goblet cells. Alcian blue staining. *GI-IM*, gastric-and-intestinal mixed type intestinal metaplasia; *I-IM*, solely intestinal type intestinal metaplasia

and colonic mucosa, but not in the gastric mucosa [19]. However, the balance of gastric and intestinal specific transcription factors may be the determinant of phenotypes of IM glands, as well as the normal alimentary tract [49]. Intestinal metaplastic cells having CA1 expression exhibit Cdx2 nuclear staining, in line with the finding by Drummond et al. [4] that Cdx2 plays an important role in the intestine-specific expression of CA1. In vitro in HeLa cells, Cdx2 exerts a positive regulatory effect by binding to a motif 87-bp upstream of the CA1 TATA box [5]. Intestinal metaplastic cells having sucrase expression also exhibit Cdx2 nuclear staining, and Suh et al. [37] have shown that intestine-specific transcription of sucrase–isomaltase, a gene that is expressed exclusively in differentiated enterocytes, is dependent on binding of Cdx2 to an evolutionarily conserved promoter element.

Several subtypes of GI-IM and I-IM can be observed in the stomachs of patients suffering from chronic gastritis [13, 26, 45, 49], and these may be considered not as independent entities, but rather as a sequence of pathological states with gradual change from stomach to intestinal character [45]. In fact, experimentally, shift from GI-IM to I-IM can be observed on sequential observation in X-ray-treated rats [54] and *Helicobacter pylori*-infected gerbils [27]. In the present study, we demonstrated decrease of MUC5AC mRNAs and increase of sucrase–isomaltase mRNAs according to the shift from isolated GI-IM to isolated I-IM by relative quantitative real-time RT-PCR at the single gland level, compatible with our previous report [49]. CA1 mRNAs also increased in line with this shift. In the pyloric mucosa, GI-IM predominates over I-IM [13, 28]. In our previous study, a total of 2,004 glandular ducts were divided phenotypically into 569 (28.4%) pyloric, 1,290 (64.4%) GI-IM, and 145 (7.2%) I-IM glands, respectively, in pyloric areas with chronic gastritis

Table 3 List of IM glands for relative quantitative RT-PCR analysis

IM types	No.	β-actin	MUC5AC	Sucrase–isomaltase	Carbonic anhydrase 1
GI	1	0	−5.87	−14.71	−9.13
	2	0	−7.62	−10.49	−8.92
	3	0	−6.88	−10.43	−7.47
	4	0	−7.46	−9.03	−6.51
	5	0	−5.8	−8.19	−5.43
	6	0	−21.71	−13.07	−4.73
	7	0	−4.83	−13.42	−3.41
	Average± SE	0	−8.60± 2.22*	−11.33± 0.92**	−6.51± 0.81***
I	1	0	−69.95	−8.78	−3.12
	2	0	−71.56	−6.96	−1.01
	3	0	−67.87	−7.16	−0.41
	4	0	−65.55	−9.03	0.58
	5	0	−65.31	−7.1	1.16
	Average± SE	0	−68.05± 1.22	−7.81± 0.45	−0.56± 0.74

Relative PCR cycle numbers shown as β-actin as an internal control
*P<0.005 vs. I-IM
**P<0.05 vs. I-IM
***P<0.005 vs. I-IM

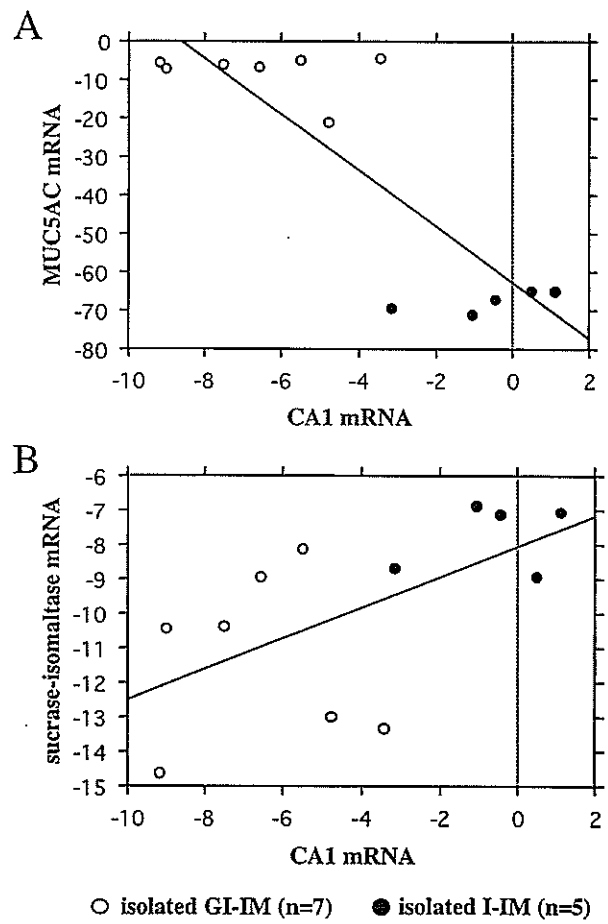

Fig. 7 Relative quantitative analysis correlation of MUC5AC, sucrase–isomaltase, and CA1 mRNAs. a Inverse correlation between the relative mRNA expression of CA1 and MUC5AC in isolated IM glands. y=−62.861−7.313×, r=0.720, P<0.05. b Correlation between the relative mRNA expression of CA1 and sucrase–isomaltase in isolated IM glands. y=−8.072+0.444×, r=0.369, P<0.05

[28]. Thus, from our present and previous data, we consider that CA1-positive IM glands may be more prone to alteration from GI-IM to I-IM compared with their CA1-negative counterparts in the pyloric region.

IM has been classified according to HID-AB staining [14], type I being considered to be virtually identical to the morphology of small intestine consisting of mucin-producing goblet cells and absorptive cells without mucin expression. Glands of types II and III are lined by goblet and columnar cells showing mild architectural distortion, respectively, with sialomucins/neutral mucins and sulphomucins in their columnar cells. Reis et al. [30] and Silva et al. [34] have analyzed expression of mucin patterns including MUC1, MUC2, MUC5AC, and MUC6 in IM glands and revealed the type I complete form to have little expression of MUC 1, MUC5AC, and MUC6 in any epithelial components, but strong expression of MUC2 in the goblet cells. In contrast, the incomplete forms of types II and III were found to express gastric markers, MUC1, MUC5AC, and MUC6, together with intestinal MUC2. Our data suggest that expression of CA1, a colonic marker, is not correlated with sulfomucin expression in type III IM, but rather is inversely related to incomplete IM expressing gastric mucins. Furthermore, CA1 was found to be more frequent in type I complete IM. On the other hand, small intestinal markers like sucrase may colocalize with gastric protein, MUC5AC, as noted with the gastric-and-intestinal-mixed phenotype.

IM is widely thought to be a precancerous lesion for differentiated type gastric cancers [1–3, 24, 36, 52, 55]. The hypothesis is based on morphological similarities between cancers and IM and on the results of comparisons of carcinomas and surrounding mucosa. However, previous studies on clonality of gastric cancers and phenotypic expression of each intestinal metaplastic or stomach cancer cells have pointed to several contradictions [8, 11, 15, 17, 23, 31, 40–44]. In addition, using microsatellite instability analysis, Tamura et al. [38] have provided evidence that the majority of differentiated adenocarcinomas of the stomach may develop through a de novo pathway. Endoh et al. [9] also made it clear that the genetic background of differentiated type tumors may differ among cellular phenotypes. In the present study, CA1 expression was observed in approximately one fourth of IM glands in the pyloric region by immunohistochemistry. We have previously shown that early stage gastric cancers, independent of the histological type, mainly consist of gastric phenotypic cancer cells, and a shift from gastric to intestinal phenotypic expression is observed with progression in experimental animal models [23, 39, 40, 41, 51, 53]. More than half of early differentiated gastric cancers have MUC5AC expression in humans [23]. However, the expression of CA1 inversely correlates with that of MUC5AC in IMs, suggesting that IM is important not as a precancerous lesion but rather as a paracancerous phenomenon [8, 18, 43]. Needless to say, many questions remain regarding the pathogenesis of IM as well as the actual relationship to gastric cancers.

In short, expression of gastric and colonic markers may be regulated in different manners, although both can be colocalized with a small intestinal marker. Although the regulatory mechanisms remain unclear, further study of stimulation and/or suppression by gastric and intestinal transcription factors appears warranted [49].

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