品摂取が少ない群と最も高い群で血清PG1/2比を比較すると，緑茶摂取群が有意に高値であった(**図1**)。50歳代では有意差はないが(**図2**)，60歳代では*H. pylori*陽性例の萎縮性胃炎の進展が抑制される可能性が示唆された。

6. 喫煙

喫煙と萎縮性胃炎に関連はないとする報告が多いが，*H. pylori*陽性の喫煙者では萎縮が進行しているという報告もある。

非喫煙群，1日1～19本，20～39本，40本以上に分けると，順に1,487/2,071例(718%)，194/283例(68.6%)，325/462例(70.3%)，117/158例(74.1%)と各群間で差はなかった。*H. pylori*陽性例で萎縮性胃炎を有する症例は，順に704/1,287例(54.7%)，78/168例(46.4%)，115/273例(42.1%)，50/102例(49.0%)であった。

Ⅲ. まとめ

緑茶摂取群では*H. pylori*陽性率が高いが，萎縮性胃炎は少なかった。*H. pylori*感染の予防効果は少ないが，感染した場合の萎縮性胃炎の進展を抑制している可能性が示唆された。

アルコール，コーヒー多飲者では*H. pylori*感染は低率であったが，萎縮性胃炎の進展には関与していなかった。味噌，漬け物など日本の伝統的発酵食品に*H. pylori*胃炎を抑制する効果はみられなかった。

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GASTROENTEROLOGY

Influence of urease activity in the intestinal tract on the results of ^{13}C-urea breath test

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Key words
^{13}C-urea breath test, *Helicobacter pylori*, intestinal bacteria, urease activity.

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Abstract

Background and Aim: A late rise in ^{13}CO2 excretion in the ^{13}C-urea breath test (UBT) should be found when the substrate passes rapidly through the stomach and makes contact with the colonic bacteria. The aim of this study was to evaluate the influence of intestinal urease activity on the results of the UBT.

Method: A total of 143 subjects who were diagnosed as *Helicobacter pylori* negative by serology, histology and rapid urease test were recruited. At the end of endoscopy, the tip of the endoscope was placed to the second part of the duodenum and 20 mL of water containing 100 mg of ^{13}C-urea was sprayed into the duodenum. Breath samples were taken at baseline and at 5, 10, 20, 30 and 60 min after administration.

Results: Of 143 subjects, breath Δ^{13}CO2 values higher than 2.5‰ were detected in six (4.2%), four (2.8%) and five (3.5%) subjects at 20, 30 and 60 min, respectively. There was no subject with high Δ^{13}CO2 values at 5 and 10 min. Only one subject had an immediate rise at 60 min.

Conclusion: Variability derived from urease activity in the intestinal tract appears to be minimal up to 60 min after ingestion of the test urea.

Introduction

^{13}C-urea breath test (UBT) has become the most convenient non-invasive method for the diagnosis of the presence of *Helicobacter pylori* (*H. pylori*) infection.[1–3] The accuracy of UBT in the diagnosis of *H. pylori* infection is high, with a sensitivity of 90–98% and a specificity of 92–100%.[1–6] The main disadvantage of the UBT is possible interference by urease activity not related to *H. pylori*, as there is bacterial flora in the mouth and the intestine. An early rise in ^{13}CO2 excretion is caused by oral urease activity, whereas a late rise should be found when the substrate passes rapidly through the stomach and makes contact with the colonic bacteria. In order to eliminate the problem of false positive results in early breath samples, some modifications of UBT have been suggested, such as mouth washing,[7,8] supplying ^{13}C-urea as a rapid-release tablet,[9,10] or breath sample collection through the nostril.[11] In addition, test meals, including citric acid, were used to slow gastric emptying and enhance the distribution of the substrate within the stomach.[3,5,12–14] Slowed gastric emptying increases the contact time between *H. pylori* urease and ^{13}C-urea, resulting in an increased breath ^{13}CO2 value.

A shorter time of breath sample collection may also be important for diagnostic value, especially for persons with rapid gastric emptying, and for avoiding false-positive results from the rapid transit of ^{13}C-urea to the colon. If intestinal bacteria have strong urease activity, a positive result can be obtained even in *H. pylori*-negative subjects. The aim of this study was to evaluate the influence of intestinal urease activity on results of UBT.

Methods

Patient recruitment

During a 2-year period, 390 consecutive patients (mean age 63.1 years, range 17–86 years, 274 females, 116 males) presenting diagnostic upper endoscopy for gastrointestinal symptoms were recruited to the study to evaluate the primary diagnosis of *H. pylori* infection. The study was approved by the local ethical committee, and prior written consent was given by all patients. All subjects had blood drawn for serological testing before endoscopy. The serum was separated, divided into aliquots, and stored at −20°C before testing. ELISA testing was performed using the EPI

HM-CAP IgG (Enteric Products, NY, USA) assay. A value of >2.2 was considered seropositive and a value of <1.9 was considered seronegative.

At endoscopy, after noting the presence and location of abnormal findings, biopsies were obtained from all seronegative subjects to diagnose *H. pylori* infection by rapid urease test (Helicocheck, Otsuka Pharmaceutical, Osaka, Japan) using one biopsy from antrum and histology (hematoxylin and eosin and Giemsa stains, one biopsy each from the greater curvature of the antrum and the midbody of the stomach). Patients were classified as infected if the rapid urease test and/or histology were positive, and they were excluded from analysis.

Subjects who had taken proton pump inhibitors, H2-receptor antagonists, antibiotics or bismuth salts in the previous 2 months were excluded from the study. Patients who had abnormal endoscopic findings, including peptic ulcers and gastric cancer, were also excluded regardless of the presence of antibody to eliminate the patient with false negative results. Finally, a total of 143 *H. pylori* negative subjects participated in the present study, including 46 females and 97 males, with a mean age of 56.9 (17–79) years.

Duodenal ^{13}C-urea breath test

At the end of endoscopy, a tip of endoscope was placed to the second part of the duodenum and 20 mL of water containing 100 mg of ^{13}C-urea was sprayed onto the duodenal mucosa using a spraying instrument. Breath samples were taken at baseline and at 5, 10, 20, 30 and 60 min after administration for analysis of the $^{13}C/^{12}C$ ratio and were expressed as delta over baseline (Δ‰). These samples were measured by isotope ratio mass spectrometry.

Results

All 143 *H. pylori*-negative subjects completed the study. Figure 1 shows the mean $\Delta^{13}CO2$ values at each time point. In general, the $^{13}CO2$ values increased gradually from the beginning, and peak enrichment values were reached after 20 min. The mean breath $\Delta^{13}CO2$ value at 20 and 60 min was 1.12 ± 0.75‰ and 1.19 ± 1.35‰, respectively.

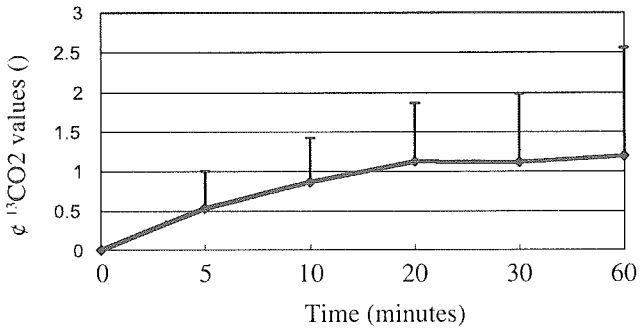

Figure 1 Time-course of changes in $\Delta^{13}CO2$ values in all 143 subjects after ^{13}C-urea was delivered directly into the second part of the duodenum through a biopsy channel using a spraying instrument. Changes in $\Delta^{13}CO2$ values after intraduodenal ingestion of ^{13}C-urea (mean ± SD).

Of 143 subjects, breath $\Delta^{13}CO2$ values higher than 2.5‰ were detected in six (4.2%), four (2.8%) and five (3.5%) subjects at 20, 30 and 60 min, respectively (Table 1). There was no patient with high $\Delta^{13}CO2$ values at 5 and 10 min after administration. Figure 2 shows changes in $\Delta^{13}CO2$ values in six subjects who had $\Delta^{13}CO2$ values higher than 2.5‰ at 20 min. The $\Delta^{13}CO2$ values were higher at 20 min than those at 30 and 60 min in all subjects. As shown in Figure 3, an immediate rise in $^{13}CO2$ excretion at 60 min was found in one of five subjects who had a $\Delta^{13}CO2$ value higher than 2.5‰ at 60 min.

Discussion

Many minor modifications of UBT have been reported, including changes in dosage of urea, type of test meals used, timing of sample collection, sampling route, and position of patients.[4–11] A Japanese standard protocol was proposed in 1998 as having high

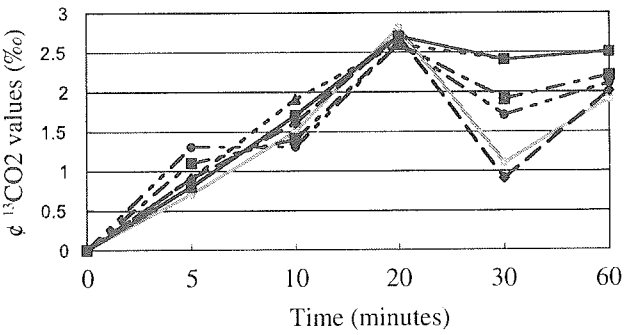

Figure 2 Changes in $\Delta^{13}CO2$ values in six subjects who had $\Delta^{13}CO2$ values higher than 2.5‰ at 20 min.

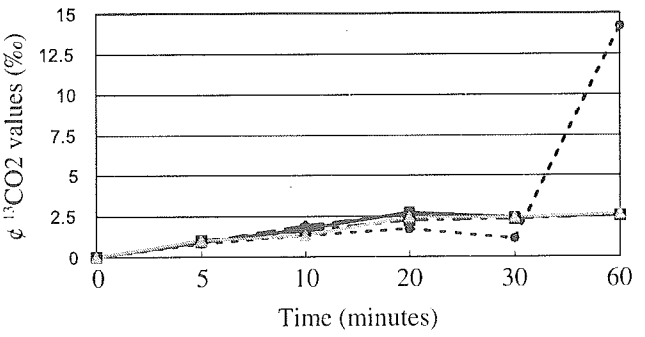

Figure 3 Changes in $\Delta^{13}CO2$ values in five subjects who had $\Delta^{13}CO2$ values higher than 2.5‰ at 60 min.

Table 1 Number of cases with $\Delta^{13}CO2$ values higher than 2.5‰ at each time point

Sampling point	Case (%)
5 min	0
10 min	0
20 min	6 (4.2%)
30 min	4 (2.8%)
60 min	5 (3.5%)

sensitivity and specificity for UBT.[1] In this method, patients who have fasted are given 100 mg of ^{13}C-urea in 100 mL water and then immediately rinse their mouth with water. After that they are placed in the left lateral decubitus position for 5 min. Breath samples are collected at baseline and at 20 min after ingestion of ^{13}C-urea. The cut-off value of standard UBT for the diagnosis of *H. pylori* infection is 2.5‰. Twenty minutes is long enough to avoid interference due to urease activity in the mouth.[7,11] Prolonging the time of sample collection to 30 min or later would only decrease the influence of oral urease activity on the results of UBT, whereas there may be a late rise in Δ^{13}CO2 values as a result of small bowel urease activity even in *H. pylori*-negative patients. According to the Japanese protocol, even when the cut-off value of standard UBT for the diagnosis of *H. pylori* infection is 2.5‰, a rise in Δ^{13}CO2 values at 20 min may be observed even in *H. pylori*-negative patients who have both rapid gastric emptying and small bowel bacterial overgrowth. From this viewpoint, information on the incidence of the urease activity that is high enough to result in false-positive UBT is thought to be important for the management of *H. pylori* infection. Therefore, we designed the present study to investigate potential interference with the UBT by urease activity in the small intestine.

We found that breath Δ^{13}CO2 values higher than 2.5‰ were detected in six (4.2%), four (2.8%) and five (3.5%) subjects at 20, 30 and 60 min, respectively. At 20-min sampling point, six subjects had Δ^{13}CO2 values higher than 2.5‰. According to the conventional protocol in Western countries, in which the breath sample is collected at 30 min after ingestion with the optimal cut-off point of 5‰, no subject had breath Δ^{13}CO2 values higher than 5‰ at 30 min. In addition, the Δ^{13}CO2 values were higher at 20 min than those at 30 and 60 min in all subjects. This suggests that a rise in Δ^{13}CO2 values at 20 min may not be caused by the rapid transit of ^{13}C-urea to the colon, but by small bowel bacterial overgrowth.

As shown in Figure 3, among five subjects who had breath Δ^{13}CO2 values higher than 2.5‰ at 60 min, an immediate rise in Δ^{13}CO2 values at 60 min was found in only one subject. This subject was considered to have rapid transit of ^{13}C-urea to the colon. This suggests that colonic urease activity does not affect the results of a Japanese standard protocol.

In the original method, *H. pylori*-negative patients with a late rise in Δ^{13}CO2 excretion at 120 min were found as a result of small bowel urease activity.[15] Actually, the microbial flora, which is dominated by *Viridans streptococci, coaglase negative Staphylococci, Haemophilus sp., Neisseria spp., Lactobacillus spp., Candida spp.* and *Aspergillus spp.*, has been demonstrated.[16,17] Fried *et al.*[18] reported that most of the bacteria identified from the duodenal aspirates belonged to species colonizing the oral cavity and pharynx, suggesting a descending route of colonization. Husebye *et al.*[17] also reported that fasting hypochlorhydria associated with gastric colonization of microbes belonging to the oro- and nasopharyngeal flora is highly prevalent in healthy old people. This suggests that a rise in Δ^{13}CO2 values at 20 min may be possibly detected in *H. pylori*-negative patients.

In the present study, breath Δ^{13}CO2 values higher than 2.5‰ were detected in six (4.2%) subjects at 20 min. If these subjects have rapid gastric emptying, false positive results of UBT may be found. Conversely, T-H Lee *et al.*[19] did not find any late rise after direct administration of the test urea into the duodenum up to 180 min in *H. pylori*-negative or -positive patients using similar procedures. However, there are some important differences between the two studies. The advantages of our study are that we: (i) studied only *H. pylori*-negative patients; (ii) studied a large number of patients; (iii) did not use a test meal, and (iv) used histology, rapid urease test and serology to diagnose *H. pylori* infection. In patients with *H. pylori* infection and frequent duodenogastric reflux, the urea solution infused into the duodenum may flow backward and make contact with *H. pylori* in the stomach. Therefore, to evaluate the influence of colonic bacterial ureolysis on the results of UBT, *H. pylori*-infected patients should be excluded from the analysis. In addition, false negative biopsies could occur when the active site of infection is missed because of the patchy distribution of *H. pylori* in the stomach. To avoid false negative results, *H. pylori* infection was defined as negativity in all of the tests (rapid urease test, histology and serology) and gave concordant negative results. In contrast, Perri *et al.*[20] demonstrated a slightly late increase in ^{13}CO2 breath excretion, possibly reflecting natural ^{13}C-enrichment of the standard meals. Based on this finding, although several protocols use a test meal to delay gastric emptying and to cause an even distribution of test urea throughout the stomach, a test meal was not used in this study.

Assuming that a large amount of test urea passes through the stomach in the blink of an eye, it is highly likely that urease activity in the intestinal tract strongly affects the UBT results. Contrary to expectation, breath Δ^{13}CO2 values higher than 2.5‰ at 20 min were detected in only six (4.2%) subjects. In a Japanese standard protocol, in which the breath sample is collected at 20 min after ingestion with the optimal cut-off point of 2.5‰, the affect of urease activity in the intestinal tract on the UBT is considered to be negligible. We conclude that variability derived from urease activity in the intestinal tract appears to be minimal up to 60 min after ingestion of the test urea.

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Editorial

How We Eradicate *H. pylori*

Kazumasa Miki*1

The progress of gastric cancer treatment started with efforts to improve diagnostic techniques for detecting early stage cancers that could be cured surgically. This ability for early detection of gastric cancer was realized by the development of double contrast radiography, the widespread use of panendoscopy utilizing small-diameter endoscopes, and the establishment of screening systems in local communities and workplaces. Gastric cancer screening using photofluorography is performed in the majority of people at regular intervals, based on the assumption that the likelihood of gastric cancer development is similar in all individuals. However, a negative result from the screening in one year does not predict the status in the next year, and there is a concern that the detection of advanced cancer may increase when people undergo screening at longer intervals.

On the other hand, there is an association between atrophic gastritis and gastric cancer known before the discovery of *Helicobacter pylori* and the concept of a high-risk group for gastric cancer has been suggested. This concept may lead to efficient detection of gastric cancer based on the assumption that there are individual variations in the likelihood of gastric cancer development.

Following the discovery of *H. pylori*, it was demonstrated that *H. pylori* infection was responsible for most cases of atrophic gastritis, and reports from many facilities showed that eradication of *H. pylori* improved atrophic gastritis. These findings suggest the possibility that *H. pylori*-negative individuals might be excluded from the high-risk group for gastric cancer. However, *H. pylori*-positive individuals may further be divided into two types: Those in one type do not show progression of atrophy, but may develop duodenal ulcers while remaining in a high-acid condition. The other type shows progression from atrophic gastritis to intestinal metaplasia and gastric cancer. It is impossible to discriminate between these two types based on serum antibody titers, urea breath test, etc., and invasive endoscopy is needed for accurate assessment. The paper by Seto presented here also demonstrates a high rate of *H. pylori* infection among patients with atrophic gastritis.

Before the discovery of *H. pylori*, we reported that serum pepsinogen (PG) values, in particular the PG 1/2 ratio, highly correlated with the extent of atrophic gastritis. We developed the serum PG method for identifying the high-risk group in gastric cancer screening, and the performance of this method so far has been satisfactory.

A further step forward has been the attempt to provide active treatment to the patients with atrophic gastritis who were found to be high risk for gastric cancer, and to help the patients move out of the high-risk group. Seto's paper emphasizes that eradication at a young age is desirable for the purpose of preventing gastric cancer development, and this point is also elaborated in Suzuki's paper. At the present, there is a controversy regarding the effectiveness of eradication in improving the cases with intestinal metaplasia developing from atrophic gastritis. From the standpoint of gastric cancer prevention, it is important that *H. pylori*-positive individuals undergo eradication before the progression of atrophic gastritis takes place. Because *H. pylori* eradication is not covered by the health insurance, it is difficult to perform this procedure in cases with atrophic gastritis. However, people are taking more interest in *H. pylori* infection, and many patients are now visiting hospitals to receive eradication. The benefit of eradication is considered to surpass the cost.

After the turn of the century, the clinical practice for gastric cancer has been undergoing a major shift in focus from early detection and treatment to the prevention of cancer development. At this point in time, the award of the Nobel Prize to Marshall and Warren for the discovery of *H. pylori* has just been announced. I wish to congratulate them, like my colleagues engaged in the study of gastritis and gastric cancer.

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Helicobacter pylori, Pepsinogen, and Gastric Adenocarcinoma in Hawaii

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Background. The objective was to investigate the association of *Helicobacter pylori* and serum pepsinogen (PG) levels with gastric adenocarcinoma.

Methods. Serum obtained from 299 patients at the time of cancer diagnosis and from 336 population-based control subjects was tested for PG I, PG II, and antibodies to *H. pylori* and to CagA.

Results. Subjects with low PG I levels or low PG I/II ratios were at increased risk for cardia and noncardia gastric cancer, whereas those with *H. pylori* or CagA seropositivity had an elevated risk for noncardia cancer only. Subjects seropositive for either *H. pylori* or CagA who had low PG I levels had the highest odds ratio (OR) (9.21 [95% confidence interval {CI}, 4.95–17.13]) for noncardia cancer, compared with subjects with neither factor. Elevated risks were also found among subjects with only 1 factor (OR, 5.40 [95% CI, 2.61–11.20] for low PG I level only; OR, 4.86 [95% CI, 5.90–8.13] for *H. pylori* or CagA seropositivity only). This pattern persisted when PG I/II ratio replaced PG I level and when CagA seropositivity alone replaced *H. pylori* immunoglobulin G or CagA seropositivity.

Conclusions. The results suggest that persons with both *H. pylori* or CagA seropositivity and a low PG I level or PG I/II ratio are highly susceptible to development of noncardia gastric cancer.

Helicobacter pylori infection has been associated with chronic superficial gastritis [1], an early step in the pathogenesis of gastric cancer [2]. *H. pylori* strains may possess a functional *cag* island, which leads to injection of CagA protein into gastric epithelial cells, where it undergoes tyrosine phosphorylation and affects signal transduction pathways [3]. Persons carrying *cagA*$^+$ *H. pylori* strains develop more-severe gastritis [4–6]. Consequently, it is important to assess CagA status when testing for *H. pylori*. Some individuals who carry *cagA*$^+$ *H. pylori* strains may have *H. pylori* antibody levels below the cutoff value and, thus, may be falsely considered to be *H. pylori* seronegative [7].

Chronic atrophic gastritis, a later step in the carcinogenic process, has been linked to a low serum pepsinogen (PG) I level or to a low serum PG I/II ratio [8]. PG I and PG II are immunologically distinct aspartic proteinases of pepsin, which digests proteins [9]. PG I is synthesized by chief cells in fundic gland mucosa, and PG II is produced in similar cells and in pyloric gland cells in the gastric antrum. Destruction of fundic glands by atrophic gastritis leads to a reduction in PG I levels and in the PG I/II ratio [10]. Even though *H. pylori* infection does not always lead to atrophic gastritis [11] and atrophic gastritis is caused by factors other than *H. pylori* infection, it is important to investigate the relationship between serum PG levels, *H. pylori* seropositivity (especially with regard to CagA status), and gastric cancer risk. Although an *H. pylori*

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Potential conflicts of interest: M.J.B. and G.I.P.-P. helped to develop the Pyloristat kit with Whittaker Bioproducts, Inc., for detection of *H. pylori*–specific serum IgG and have a royalty interest in the product. However, this kit is currently licensed by the US Food and Drug Administration and is available for clinical or research use by any physician or researcher. Furthermore, the purpose of the present study was not to assess the validity of the test (this has previously been done by the manufacturer) but to use a standard kit to conduct the study. Therefore, it is not believed that there is a conflict of interest. M.J.B. also discloses that, as a discoverer of *cagA*, he may receive royalties from licenses at Vanderbilt University.

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infection followed by atrophy is considered to be a common pathway to gastric cancer [12], these are not proven necessary factors.

Four case-control studies, in which serum samples were obtained at the time of diagnosis of gastric cancer [8, 13–15], and 3 cohort studies [10, 16, 17] have evaluated serum PG levels, *H. pylori* status, and gastric cancer risk, but the results are inconsistent. Because of these equivocal findings and because only 1 previous study determined CagA status [15], we conducted a case-control study of gastric cancer in Hawaii that included measurement of levels of serum PGs and antibodies to *H. pylori* and to CagA. Our hypothesis was that stratification for both *H. pylori*/CagA and location of the cancer would permit a clearer understanding of the relationship between *H. pylori* infection, PG levels, and gastric cancer risk.

SUBJECTS, MATERIALS, AND METHODS

Study population. Patients initially received diagnoses of gastric cancer between September 1993 and April 1999 at 8 major hospitals on the island of Oahu and were identified by the Hawaii Tumor Registry, a member of the Surveillance, Epidemiology, and End Results program of the National Cancer Institute [18]. There were 591 patients with tissue-confirmed gastric cancer, whose pathology reports and histologic slides were reviewed by the study pathologist (G.N.S.). Of these 591, 59 had a diagnosis of gastric lymphoma or malignant stromal tumor and were excluded from the analysis. Because we were limited to using unused preoperative serum available from participating hospitals, 299 (56%) of the remaining 532 patients with gastric adenocarcinoma had a sample available for the study; these serum samples were obtained at the time of diagnosis. The patients were 26–97 years of age, were residents of Oahu, and belonged to 1 of 6 ethnic groups: white, Chinese, Filipino, Japanese, Korean, or Native Hawaiian (including part Hawaiian). Ethnicity was based on having at least 3 of 4 grandparents belonging to the same ethnicity, unless it included Hawaiian heritage.

Eligible control subjects were identified from lists of Oahu residents who had been interviewed by the Health Surveillance Program of the Hawaii Department of Health. Each year, this program identifies a 1% random sample of all households in the state, with a sampling procedure modeled after that of the National Health Survey [19]. To supplement the pool of eligible control subjects ≥65 years of age, Oahu residents registered with the Health Care Financing Administration (now the Centers for Medicare and Medicaid Services) also were identified; this institution is estimated to service 95% of individuals ≥65 years of age in the United States [20].

The population-based control subjects were frequency matched to the case patients on the basis of sex, ethnicity, and 5-year age groups. A total of 759 potential control subjects without a history of gastric cancer were identified. Of these, 36 (5%) had died or were too ill to be questioned. Among the remaining 723 potential control subjects, 220 (30%) refused the interview, and 57 (8%) were unlocatable, had moved, or had difficulty communicating in English. Interviews were completed for the remaining 446 control subjects. Of these, 336 (75%) provided blood samples, whereas 84 (19%) refused and 26 (6%) did not provide blood samples for other reasons.

Pathologic classification. The histologic classification of hematoxylin-eosin–stained slides used for this study was that of Lauren [21], which classifies gastric adenocarcinomas into the following basic types: intestinal, diffuse, and mixed/other. In all, there were 212 (71%) intestinal, 65 (22%) diffuse, and 22 (7%) mixed/other cases.

The identification of subsite was based on the gross description of cancers in resection specimens. Tumors were classified as "cardia" if the cardioesophageal junction was involved. The noncardia (distal) cases included the corpus if it arose in the oxyntic mucosa, including the mucosa of the fundus, and included the antrum if it involved the pyloric antrum, including the antrocorpus junction. There were 35 cardia and 264 noncardia cases in the study.

Serologic methods. The blood samples from case patients and control subjects were tested for the following: serum PG I, serum PG II, and IgG antibodies to *H. pylori* and to the CagA protein of *H. pylori*. The measurement of serum PG level was performed by immunoradiometric assay [22] using PG I, II RIA BEAD kits (Dainobot). Low PG levels were defined as PG I levels ≤30 ng/mL or PG I/II ratios ≤2.0. These cut points best discriminated between case patients and control subjects in our study; other studies have used different definitions of low PG levels, based on their study populations [8, 9, 10, 14–17].

Assays for serum IgG antibodies to *H. pylori* group antigens and to a recombinant fragment of the CagA protein were performed exactly as described in prior studies [23, 24]. All assays were performed at least in duplicate on coded specimens, by laboratory personnel blinded to the identity or status of the study subjects. A total of 227 case patients had both the PG and *H. pylori*/CagA tests performed. Because of limited amounts of sera, 49 had only the *H. pylori*/CagA tests performed, and 23 had only the PG tests performed. All 336 control subjects had the *H. pylori*/CagA tests performed, and 334 had the PG tests performed.

Statistical analysis. For the statistical analysis, we used unconditional logistic regression to compute odds ratios (ORs) and 95% confidence intervals (CIs) for exposures of interest [25]. Indicator measures representing PG I or PG I/II status and *H. pylori* or CagA status were entered as independent variables. The models were adjusted for the variables used in the frequency match: sex, ethnicity, and age (continuous). Separate

models were estimated for cardia and noncardia cancers and for intestinal and diffuse cancers, to determine whether the risk factors varied by cancer subsite or histologic type; all control subjects were used in these models.

A joint-effects analysis was performed and is defined as assessment of the risk for gastric cancer among subjects with *H. pylori* or CagA seropositivity who have low PG values, as well as among subjects abnormal for only 1 of these factors, compared with subjects with neither factor. Because it is not expected that both factors together equal the sum of the individual factors, on either an additive or a multiplicative scale, a test of interaction is not presented in table 3.

RESULTS

Study population. There were 299 case patients with gastric adenocarcinoma and 336 control subjects in the study. The mean (±SD) age was 70.7 (±11.8) years for case patients and 70.6 (±12.7) years for control subjects. There were a total of 183 male and 116 female case patients. Of the control subjects, 228 were men and 108 were women. Because results were similar for men and women, they were combined in the analyses.

Serum PGs. We compared 250 case patients with gastric adenocarcinoma and 334 control subjects according to the percentage with low serum PG I levels or low serum PG I/II ratios (table 1). The ORs for gastric cancer were significant for both a low PG I level (OR, 2.66 [95% CI, 1.81–3.90]) and a low PG I/II ratio (OR, 2.78 [95% CI, 1.84–4.21]). After separation by tumor location, there were 221 noncardia (distal) cases and only 29 cardia cases. A significant association was observed for both subsites. The cases were also separated by histologic type (intestinal or diffuse); there was a positive association of a low PG I level and a low PG I/II ratio with both types of tumor.

H. pylori or CagA status. A similar analysis was performed for 276 case patients and 336 control subjects according to *H. pylori* or CagA status (table 2). More case patients than control subjects were seropositive for either *H. pylori* or CagA (OR, 2.82 [95% CI, 1.99–3.99]). Similarly, more case patients than control subjects were seropositive for CagA alone (OR, 2.59 [95% CI, 1.84–3.65]). Next, the cases were separated by tumor location. The positive association persisted for noncardia cases (OR, 3.41 for *H. pylori* or CagA seropositivity; OR, 3.16 for CagA seropositivity alone) but not for cardia cases of the stomach.

When the analysis in table 2 was limited to only subjects who were *H. pylori* seropositive instead of subjects who were *H. pylori* or CagA seropositive, the OR for the noncardia cases was 2.49 (95% CI, 1.76–3.54) instead of 3.41, reflecting the fact that there were 24 case patients and 14 control subjects who were *H. pylori* seronegative and CagA seropositive.

Because of the lack of a positive association between *H. pylori* seropositivity and cardia cancer found in this study and in prior studies [23, 24, 26], only noncardia cases were included in subsequent analyses. When they were separated according to histologic type (table 2), the associations with being seropositive for either *H. pylori* or CagA or with being seropositive for CagA alone were stronger for the diffuse noncardia cases.

Joint effect of H. pylori/CagA seropositivity and PG values. To evaluate the joint effect of *H. pylori* IgG or CagA seropositivity and low PG I level on gastric cancer risk, we defined those who were *H. pylori* and CagA seronegative and had normal PG I levels as the referent group (table 3). Compared with the referent group, subjects who were *H. pylori* and CagA seronegative and had low PG I levels had an OR of 5.40 (95% CI, 2.61–11.20) for noncardia gastric cancer. Subjects who were seropositive for either *H. pylori* or CagA and had normal PG I levels had an OR of 4.86 (95% CI, 2.90–8.13), whereas those who were seropositive for either *H. pylori* or CagA and had low PG I levels had an OR of 9.21 (95% CI, 4.95–17.13). A

Table 1. Odds ratios (ORs) for gastric cancer by pepsinogen (PG) I level and PG I/II ratio, according to subsite location and histologic type of gastric cancer.

		PG I		PG I/II	
Group	Total no.	No. (%) with low level[a]	Adjusted OR[b] (95% CI)	No. (%) with low ratio[c]	Adjusted OR[b] (95% CI)
Control subjects	334	65 (19.5)	1.00	49 (14.7)	1.00
Case patients	250	96 (38.4)	2.66 (1.81–3.90)	80 (32.0)	2.78 (1.84–4.21)
Cancer subsite					
Cardia	29	10 (34.5)	2.41 (1.02–5.69)	8 (27.6)	2.57 (1.01–6.55)
Noncardia	221	86 (38.9)	2.69 (1.81–3.99)	72 (32.6)	2.85 (1.85–4.37)
Cancer histologic type					
Intestinal	177	72 (40.7)	2.65 (1.75–4.02)	60 (33.9)	2.79 (1.79–4.37)
Diffuse	55	20 (36.4)	3.35 (1.68–6.71)	13 (23.6)	2.51 (1.16–5.44)

NOTE. CI, confidence interval.

[a] A low PG I level was defined as ≤30 ng/mL.

[b] Adjusted for sex, ethnicity, and age.

[c] A low PG I/II ratio was defined as ≤2.0.

Table 2. Odds ratios (ORs) for gastric cancer by *Helicobacter pylori* and CagA status, according to subsite location and histologic type of gastric cancer.

Group	Total no.	*H. pylori* or CagA		CagA only	
		No. (%) seropositive	Adjusted OR[a] (95% CI)	No. (%) seropositive	Adjusted OR[a] (95% CI)
Control subjects	336	164 (48.8)	1.00	97 (28.9)	1.00
Case patients	276	199 (72.1)	2.82 (1.99–3.99)	138 (50.4)	2.59 (1.84–3.65)
Cancer subsite					
Cardia	33	14 (42.4)	0.97 (0.45–2.09)	4 (12.1)	0.40 (0.13–1.18)
Noncardia	243	185 (76.1)	3.41 (2.35–4.94)	134 (55.1)	3.16 (2.22–4.51)
Cancer histologic type[b]					
Intestinal	172	126 (73.3)	2.72 (1.82–4.08)	93 (54.1)	2.82 (1.92–4.15)
Diffuse	53	46 (86.8)	11.30 (4.48–28.48)	31 (58.5)	5.11 (2.56–10.21)

NOTE. CI, confidence interval.

[a] Adjusted for sex, ethnicity, and age.

[b] Excludes cardia cases.

similar pattern persisted when noncardia cases were separated into intestinal and diffuse histopathologic categories. For each type, subjects who were seropositive for either *H. pylori* or CagA and had low PG I levels had the highest ORs; for diffuse cancers, the OR for that group was >40 (95% CI, 9.51–174.60).

When PG I/II ratios were examined instead of PG I levels, subjects who had low ratios and were seropositive for either *H. pylori* or CagA were found to have the highest OR (6.88 [95% CI, 3.81–12.44]) for noncardia cancer. The same pattern was seen for intestinal cases but not for diffuse cases, possibly because of their small sample size.

Next, we defined subjects who were CagA seronegative and had normal PG I levels as the referent group (table 3). Compared with the referent group, subjects who were CagA seronegative and had low PG I levels had an OR of 4.76 (95% CI, 2.72–8.34) for noncardia cancer. Subjects who were CagA seropositive and had normal PG I levels had an OR of 4.79 (95% CI, 3.00–7.63), and those who were CagA seropositive and had low PG I levels had an increased OR, of 6.40 (95% CI, 3.45–11.89). Again, for the intestinal and diffuse types, subjects who were CagA seropositive and had low PG I levels had the highest ORs. Finally, when PG I/II ratios were used instead of PG I levels, those who had low PG I/II ratios and were seropositive for CagA were found to have the highest OR (6.08 [95% CI, 3.22–11.48]) for noncardia cancer.

DISCUSSION

In the present study, subjects with low PG I levels or low PG I/II ratios were at an increased risk for cardia and noncardia cancer of the stomach, including both histologic types, as shown in a previous study [8]. Subjects with *H. pylori* or CagA seropositivity had an elevated risk of intestinal and diffuse cancer of the distal (noncardia) stomach, but *H. pylori* seropositivity was not associated with cancer of the cardia. This is consistent with findings of earlier investigations [23, 24, 26]. When we limited the analysis shown in tables 1 and 2 to the 227 case patients who had both PG and *H. pylori*/CagA tests performed, the results were very similar (data not shown).

It should be noted that 44% of the identified case patients and 56% of the potential control subjects did not participate in this study. As a result, selection bias is possible, although there is no a priori reason to suspect the presence of bias in the analyzed data. In addition, there can be inaccuracies in measuring *H. pylori* status and PG levels at the time of diagnosis, but such inaccuracies (which would cause misclassification) generally would lead to weakening rather than strengthening of the associations found in this study.

Our results support those of 2 case-control studies that showed a positive association of gastric cancer with a low PG I level, a low PG I/II ratio, and *H. pylori* seropositivity [8, 13]. In another study, a low PG I level or a low PG I/II ratio—but not *H. pylori* seropositivity—was associated with an increased risk for gastric cancer [14]. Other investigators found that, among subjects with low PG I/II ratios, *H. pylori* antibody levels and CagA seropositivity had an inverse instead of a positive association with gastric cancer [15]. Among those with normal PG I/II ratios in the same study, there was no association with *H. pylori* and CagA seropositivity. The authors suggested that extensive fundic mucosal atrophy caused a loss of *H. pylori* with a consequent reduction in antibody titer. Endoscopic studies have shown that the presence of *H. pylori* in the stomach diminishes with the progression of gastric damage, characterized by atrophy, intestinal metaplasia, and dysplasia [27, 28].

When we also separated our study subjects into those with low PG I/II ratios and those with normal PG I/II ratios, we found that, among those with low ratios, there was no significant association between *H. pylori* seropositivity and noncardia gastric cancer (OR, 1.49 [95% CI, 0.60–3.74]) or between CagA seropositivity and noncardia gastric cancer (OR, 1.64 [95% CI, 0.74–3.66]). These results support the view that atrophic gas-

Table 3. Adjusted odds ratios (ORs) for combinations of pepsinogen (PG) I levels, PG I/II ratios, and *Helicobacter pylori* and CagA status, according to histologic types of noncardia gastric cancer.

Clinical group	All noncardia cancer cases		Intestinal cancer		Diffuse cancer	
	No. of case patients/no. of control subjects	Adjusted OR[a] (95% CI)	No. of case patients/no. of control subjects	Adjusted OR[a] (95% CI)	No. of case patients/no. of control subjects	Adjusted OR[a] (95% CI)
H. pylori/CagA seronegative, PG I level normal	24/144	1.00	19/144	1.00	3/144	1.00
H. pylori/CagA seronegative, PG I level low	22/26	5.40 (2.61–11.20)	19/26	5.06 (2.34–10.97)	3/26	8.92 (1.48–53.65)
H. pylori or CagA seropositive, PG I level normal	99/125	4.86 (2.90–8.13)	64/125	3.64 (2.05–6.45)	25/125	14.84 (4.04–54.47)
H. pylori or CagA seropositive, PG I level low	55/39	9.21 (4.95–17.13)	40/39	6.91 (3.53–13.53)	13/39	40.74 (9.51–174.60)
H. pylori/CagA seronegative, PG I/II ratio normal	34/156	1.00	28/156	1.00	4/156	1.00
H. pylori/CagA seronegative, PG I/II ratio low	12/14	4.22 (1.75–10.18)	10/14	3.54 (1.39–9.05)	2/14	8.25 (1.16–58.85)
H. pylori or CagA seropositive, PG I/II ratio normal	105/129	3.77 (2.38–5.98)	64/129	2.57 (1.54–4.20)	33/129	15.05 (4.80–47.23)
H. pylori or CagA seropositive, PG I/II ratio low	49/35	6.88 (3.81–12.44)	40/35	5.78 (3.09–10.81)	5/35	12.58 (2.84–55.62)
CagA seronegative, PG I level normal	47/198	1.00	31/198	1.00	12/198	1.00
CagA seronegative, PG I level low	41/39	4.76 (2.72–8.34)	34/39	5.07 (2.76–9.31)	6/39	4.32 (1.33–14.03)
CagA seropositive, PG I level normal	76/71	4.79 (3.00–7.63)	52/71	4.52 (2.66–7.67)	16/71	5.62 (2.27–13.92)
CagA seropositive, PG I level low	36/26	6.40 (3.45–11.89)	25/26	5.59 (2.82–11.10)	10/26	14.36 (4.75–43.41)
CagA seronegative, PG I/II ratio normal	61/209	1.00	42/209	1.00	15/209	1.00
CagA seronegative, PG I/II ratio low	27/28	3.53 (1.90–6.57)	23/28	3.55 (1.82–6.91)	3/28	2.31 (0.55–9.70)
CagA seropositive, PG I/II ratio normal	78/76	3.67 (2.37–5.69)	50/76	3.07 (1.87–5.04)	22/76	5.93 (2.64–13.34)
CagA seropositive, PG I/II ratio low	34/21	6.08 (3.22–11.48)	27/21	6.18 (3.14–12.17)	4/21	5.77 (1.52–21.90)

NOTE. CI, confidence interval.

[a] Adjusted for sex, ethnicity, and age.

tritis reduces *H. pylori* colonization. Concurrently, among those with normal PG I/II ratios, there was a positive association of *H. pylori* seropositivity (OR, 2.65 [95% CI, 1.73–4.08]) and CagA seropositivity (OR, 3.58 [95% CI, 2.31–5.56]) with noncardia gastric cancer, which is consistent with results from prior studies showing a positive association of *H. pylori* seropositivity with gastric cancer risk [8, 13].

In our study, there were a total of 24 (9% of 276) patients with gastric adenocarcinoma and 14 (4% of 336) control subjects who had anti-CagA antibodies in their sera, even though their *H. pylori* IgG antibody test results were negative. We have recently confirmed that some persons who are culture positive for *H. pylori* do not produce sufficiently high antibody levels to antigens present in the whole-cell ELISA to be designated as *H. pylori* seropositive but do meet the threshold for anti-CagA antibodies [7]. Because of this phenomenon, it is important to test for antibodies to both CagA and *H. pylori* in epidemiologic studies, to determine potential associations of *H. pylori* (especially *cagA*$^+$ strains) with conditions of interest.

The relatively high prevalence of CagA seropositivity with *H. pylori* seronegativity (9%) in patients with gastric cancer is consistent with earlier observations that this discordance is most pronounced in groups with substantial atrophic gastritis [29]. If confirmed, these findings suggest that CagA seropositivity is retained longer after the development of atrophic gastritis than is a diagnostic *H. pylori* titer.

Cohort studies have the advantage of obtaining serum samples before illnesses are diagnosed. Consequently, it is more certain in cohort studies that low PG levels or *H. pylori* seropositivity preceded the diagnosis of gastric cancer. Furthermore, the effect of the time interval between collection of serum specimens and diagnosis can be assessed. In a cohort study in Japan, Watanabe et al. reported that atrophic gastritis, identified by both a low PG I level and a low PG I/II ratio, was a more important risk factor for gastric cancer than was *H. pylori* infection [16]. Their results suggested that *H. pylori* infection has an indirect relationship with gastric cancer, through the development of atrophic gastritis.

Researchers in Finland reported that a low PG I level had a stronger association with gastric cancer than did elevated levels of serum anti–*H. pylori* IgG [17]; the association of low PG I levels with cancer was stronger at shorter follow-up times, whereas the association of *H. pylori* with cancer became stronger with longer intervals until diagnosis. In addition, they showed that low PG I levels were associated with elevated gastric cancer risk, regardless of *H. pylori* antibody status.

In contrast, a cohort study in California [10] reported that low PG I levels without evidence of *H. pylori* infection was not associated with cancer (OR, 0.8), whereas *H. pylori* infection in the absence of low PG I levels was independently associated with cancer (OR, 2.4). For subjects in whom both *H. pylori* infection and low PG I levels were present, there was a marked increase in risk for distal gastric cancer (OR, 10.0). The differing time interval between serum collection and cancer diagnosis—24 years in the California study and 13 years in the Finnish study—might explain their divergent results, since extensive replacement of fundic glands by intestinalized mucosa creates an unfavorable environment for *H. pylori* [27, 28]. Serum from the California subjects was more likely to be obtained before the process of intestinalization was extensive.

Our results showed that the magnitude of the ORs was similar for low PG I levels or PG I/II ratios and *H. pylori* or CagA seropositivity. When we studied the joint effect of PG I levels and PG I/II ratios with *H. pylori* and CagA status among patients with noncardia cancer, there was an accentuation in risk for those who had a low PG I level or PG I/II ratio, regardless of *H. pylori*/CagA status, as well as for those who had a normal PG I or PG I/II ratio and who had *H. pylori* or CagA seropositivity. The subjects in our study with noncardia cancer had extensive replacement of their fundic mucosa by intestinalized mucosa [30], so that our results were similar to those of the Finnish cohort study [17].

Nonetheless, the highest risk for noncardia gastric cancer was observed among those with either *H. pylori* or CagA seropositivity and a low PG I level or a low PG I/II ratio in both cohort studies [10, 17], as well as in the present study. Although both *H. pylori* tests and PG levels measured at the time of diagnosis have their limitations, these results suggest that persons seropositive for *H. pylori*, especially for *cagA*$^+$ strains, who also develop atrophic gastritis are highly susceptible to noncardia gastric cancer. Having either factor alone also led to a smaller increase in risk; this increase may have been accentuated by false-negative results in these groups, especially in the low-PG group. *H. pylori* can induce a continuous inflammatory process in the stomach [12], which begins as superficial gastritis and can evolve in time into the extensive atrophic gastritis that is characterized by a low serum PG I level or a low PG I/II ratio. The risk for noncardia cancer is proportionate to the surface area of the metaplasia [31]. These results also emphasize the heightened risk for the diffuse histologic type of noncardia gastric cancer that is associated with atrophy and *cagA*$^+$ *H. pylori* strains (table 3). This strong association has not, to our knowledge, been reported elsewhere and needs to be confirmed by others.

Separation of gastric cancer into cardia and noncardia cases is important in assessing the true association of risk factors with this disease. The incidence of noncardia gastric cancer, which has been the predominant gastric cancer in the United States, is steady or declining, whereas the incidence of cardia cancer has been increasing [32, 33]. Our results suggest that cardia cancer is associated with a low PG I level or a low PG

I/II ratio but that *H. pylori* does not appear to contribute to its development.

In conclusion, persons with both *H. pylori* or CagA seropositivity and a low PG I level or a low PG I/II ratio in the present study were at high risk for noncardia cancer. Since cardia cancer was associated with low PG I levels or low PG I/II ratios but not with *H. pylori* seropositivity, separation of gastric cancer into cardia and noncardia subsites is important in identifying risk factors associated with these disease entities.

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Original Article

Coexistence of gastric- and intestinal-type endocrine cells in gastric and intestinal mixed intestinal metaplasia of the human stomach

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Intestinal metaplasia (IM) in the human stomach has previously been classified into a gastric and intestinal mixed (GI-IM) and a solely intestinal phenotype (I-IM). The phenotypes of mucous and endocrine cells were evaluated in 3034 glandular ducts associated with chronic gastritis. In the pyloric region, the relative expression of gastric endocrine cell markers, such as gastrin and somatostatin, decreased gradually from glandular ducts with only gastric mucous cell phenotype (G type) to GI-IM toward I-IM, while that of the intestinal endocrine cell markers, glicentin, gastric inhibitory polypeptide (GIP), and glucagon-like peptide-1 (GLP-1) was inversely correlated. In the fundic region, gastrin-positive cells emerged in the pseudo-pyloric and GI-IM glands, whereas I-IM glands did not possess any gastrin-positive cells, suggesting the presence of a distinct pathway of intestinalization. Double staining revealed coexistence of gastrin- and GLP-1-positive cells in the same gland and occasionally in the same cell in GI-IM glands. These results suggest that the phenotypes of endocrine cells are in line with those for mucous counterparts and support the concept that all of the different types of mucous and endocrine cells in normal and IM glands might be derived from a single progenitor cell in each gland.

Key words: endocrine cell, gastric and intestinal mixed intestinal metaplasia, pseudopyloric metaplasia, stomach

Intestinal metaplasia (IM) is common in the human stomach. It occurs as a result of *Helicobacter pylori* (*H. pylori*) infection and consequent chronic gastritis.[1,2] The presence of IM has been thought to increase the risk of gastric cancer.[3–7] But many questions remain regarding its pathogenesis to neoplasias. Several classifications of IM have been suggested by pathologists. Kawachi *et al.* first proposed division into complete and incomplete types on the basis of morphology.[8] Jass and Filipe described three grades of IM with classical mucin staining.[9] Although these classifications are generally accepted, they are based upon only intestinal properties and do not consider gastric properties that are still preserved in association.[10] We have therefore proposed a new classification of IM on the basis of cellular differentiation status using both gastric and intestinal mucous cell markers with division into gastric and intestinal mixed phenotype IM (GI-IM) and solely intestinal phenotype IM (I-IM).[11] Downregulation of *Sox2*, as well as ectopic expression of *Cdx* genes, are important factors for the development of IM.[12] Experimentally, the alternation of phenotypes of IM can be clearly observed on sequential observation in X-ray-treated rats[13] and *H. pylori*-infected gerbils.[14] However, in the human there are many unsolved problems regarding the differences between GI-IM and I-IM.

Regarding the cellular differentiation of endocrine cells in the gastrointestinal tract, gastrin and somatostatin are predominantly detectable in the pyloric glands of the stomach, while a product of glicentin, gastric inhibitory polypeptide (GIP), and glucagon-like peptide-1 (GLP-1) are characteristic of the small intestine and colon.[15–18] Therefore, gastrin and somatostatin could be gastric endocrine cell markers, while glicentin, GIP, and GLP-1 could be intestinal ones. Glicentin is an enteroglucagon with tropic actions on the intestinal epithelial cells,[19,20] while GIP exhibits a glucose-dependent insulinotropic effect,[21] and GLP-1 influences gastric motility and sensation.[22] Several studies have shown that change of endocrine cells is observed in IM in the pyloric mucosa.[20,23,24] However, it is still not clear what exactly occurs in phenotypes of endocrine cells in association with those of gastric mucous

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cells, particularly in GI-IM. Furthermore, there has not been reported intestinalization of mucous epithelium in association with the phenotypic change of endocrine cells in the fundic regions.

In the present study we therefore focused on the intestinalization of endocrine cells in the light of our classification of IM through GI-mixed toward I-IM in both pyloric and fundic mucosa.

MATERIALS AND METHODS

Samples

A total of 25 stomachs, resected due to gastric cancer at Aichi Cancer Center Hospital, were investigated. Of the 25 cases, seven were of early gastric cancer and 18 were of advanced gastric cancer. They were divided histologically into two well-differentiated, 13 moderately differentiated, eight poorly differentiated, and two signet-ring cell carcinomas. The group consisted of 17 men and eight women, aged 63.6 ± 9.0 years. Eleven samples excluding invasion were taken from the fundus and 14 from the pylorus, which were >5 mm distant from gastric cancers. All 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 (HE) for routine histological assessment. Control samples of normal gastrointestinal tract were taken from stomach, duodenum, jejunum, ileum, and colon in three cases surgically resected at Aichi Cancer Center Hospital. Of the three cases, one was from a 68-year-old man, and two were from women (37 years old and 61 years old, respectively). Two pathologists evaluated the histological slides independently (TT and KI).

Mucin histochemistry and immunohistochemistry

For mucin histochemistry, we adopted paradoxical concanavalin A staining (PCS) for identifying class III mucin in mucous neck cells and pyloric glandular epithelium.[25,26] Expression of human gastric mucin (HGM) and mucin core protein (MUC)2, chromogranin A, gastrin, somatostatin, glicentin, GIP, and GLP-1 was examined using specific antibodies (Table 1). The precise procedures for immunohistochemical demonstration were as previously described.[11,27–29]

Phenotypic classification of glandular ducts in the stomach with reference to mucous cell markers

All of the straight glandular ducts from 25 stomach sections were evaluated and divided histologically and phenotypically into six types: pyloric glandular duct (P), fundic glandular duct (F), pseudopyloric glandular duct in fundic mucosa (pseudo-P), gastric and intestinal mixed phenotype IM (GI-IM), solely intestinal phenotype IM without Paneth cells (I-IM-Pa(–)), and solely intestinal phenotype IM with Paneth cells (I-IM-Pa(+)) using the gastric and intestinal mucous cell markers.[11,27] P ducts and pseudo-P ducts, being positive for PCS, have surface mucous cells stained with HGM, but are negative for an intestinal mucous cell marker, MUC2. The F duct similarly stains with HGM in surface mucous cells and PCS in mucous neck cells, again being negative for MUC2. The GI-IM exhibits staining for at least one gastric mucous cell marker as well as MUC2 intestinal mucous cell marker, whereas I-IM are positive for MUC2, with no staining for gastric mucous cell markers. Regarding I-IM, we distinguish between lesions with and without Paneth cells. GI-IM and I-IM-Pa(–) belong to the incomplete IM category while I-IM-Pa(+) corresponds to complete-type IM. We have judged the phenotypes of 1030 fundic and 2004 pyloric glandular ducts in areas of chronic gastritis. Ducts with irregular shape or branching were excluded from the analysis.

Phenotypic classification of endocrine cells in the stomach with reference to endocrine cell markers

The endocrine cells of glandular ducts were identified as positive for chromogranin A. Gastrin and somatostatin are markers of the gastric endocrine cell, whereas glicentin, GIP,

Table 1 Antibodies for immunohistochemistry

Antibodies	Clonality	Dilution	Source
Anti-human chromogranin A (344–374)	Polyclonal	1:1000	Y
Anti-human gastrin 34 (1–15)	Polyclonal	1:2000	Y
Anti-human somatostatin	Polyclonal	1:200	D
Anti-human glicentin (1–32)	Polyclonal	1:2000	Y
Anti-human gastric inhibitory polypeptide	Polyclonal	1:5000	Y
Anti-human glucagon-like peptide-1 (7–36)	Polyclonal	1:5000	Y
Anti-human gastric mucin (clone 45M1)	Monoclonal	1:200	N
Anti-human MUC2 (clone Ccp58)	Monoclonal	1:100	N

D, DakoCytomation, Glostrup, Denmark; N, Novocastra, Newcastle upon Tyne, UK; Y, Yanaihara Institute, Fujinomiya, Japan.

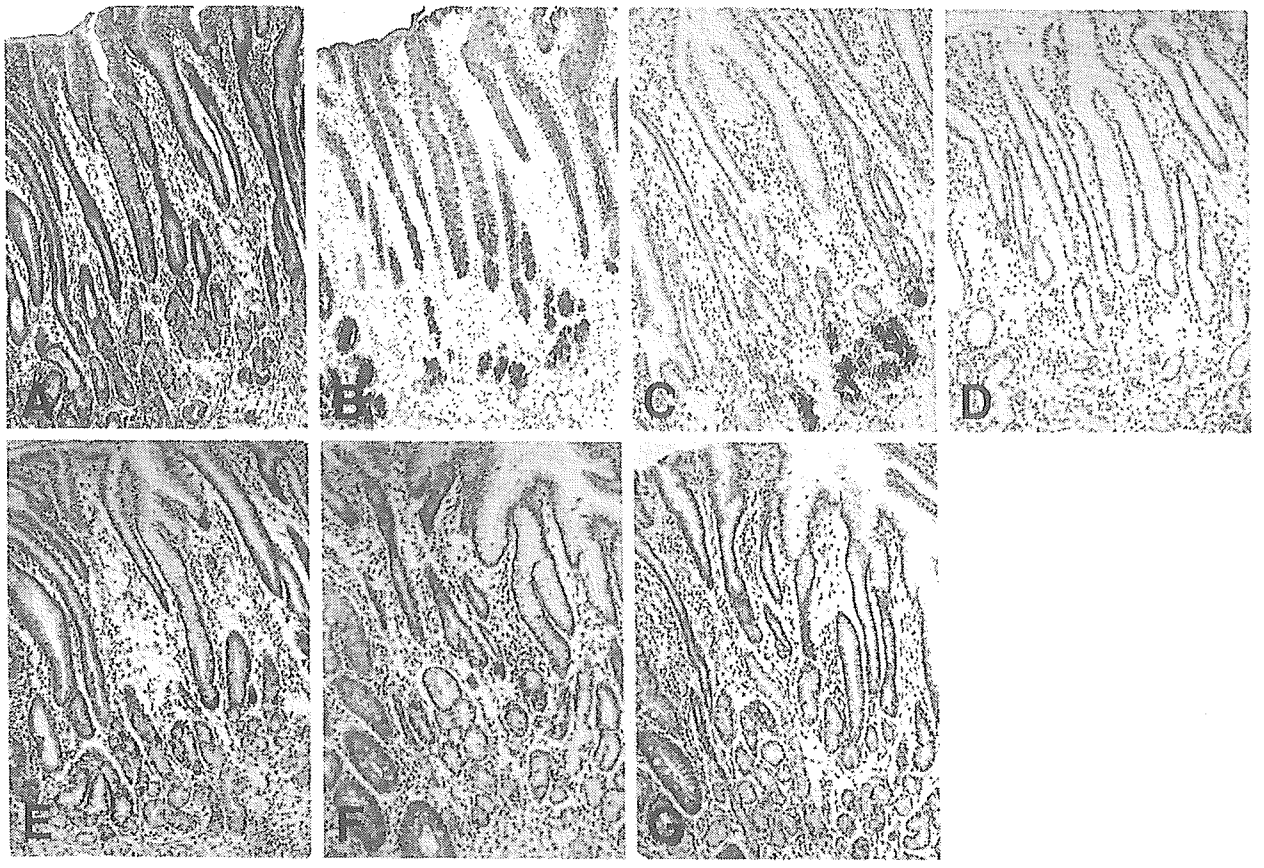

Figure 1

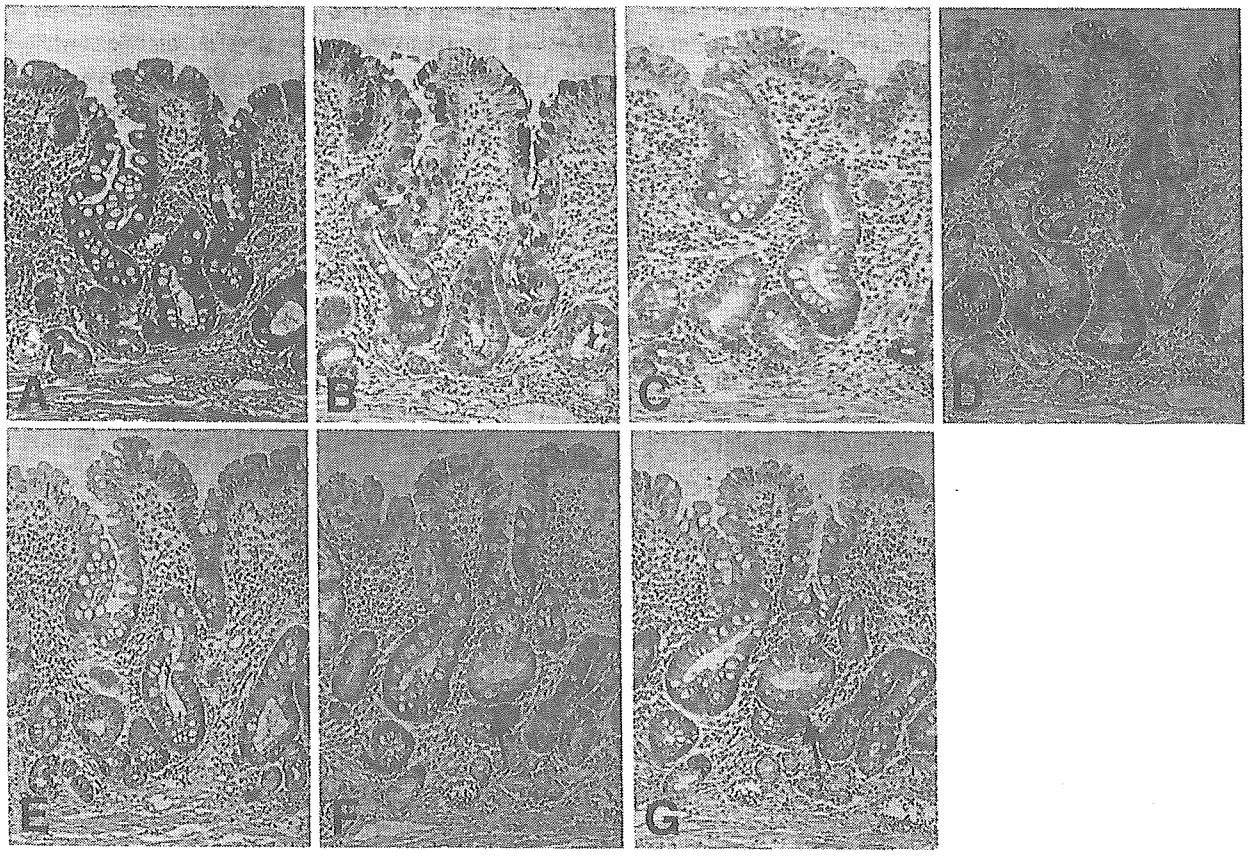

Figure 2

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

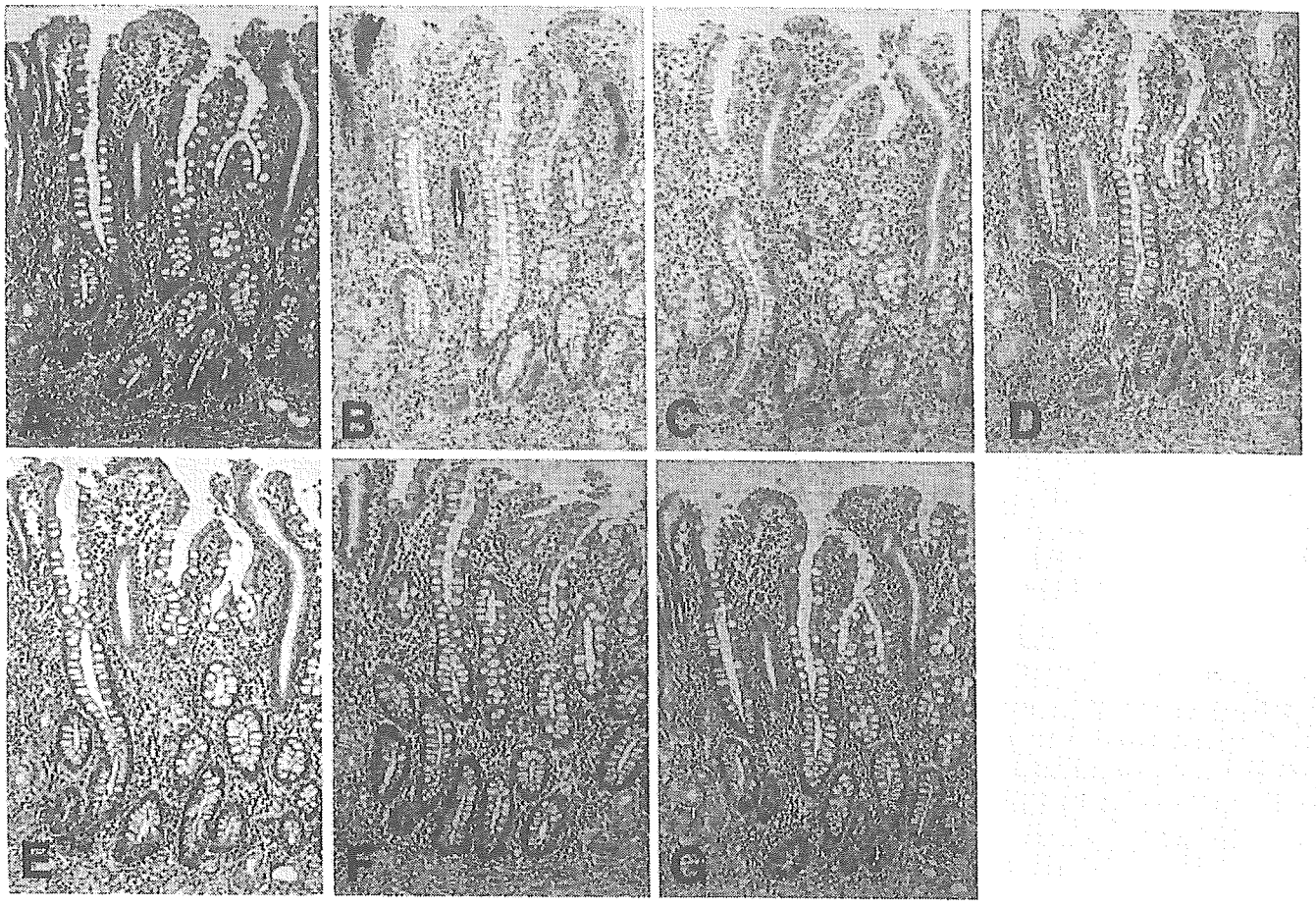

Figure 3 Solely intestinal phenotype of intestinal metaplasia (IM) with Paneth cells (I-IM-Pa(+)). (**A**) HE staining. (**B**) Note lack of human gastric mucin (HGM) in the cytoplasm of the I-IM-Pa(+) glands. (**C**) No staining of paradoxical concanavalin A in the cytoplasm of I-IM-Pa(+) glandular 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.

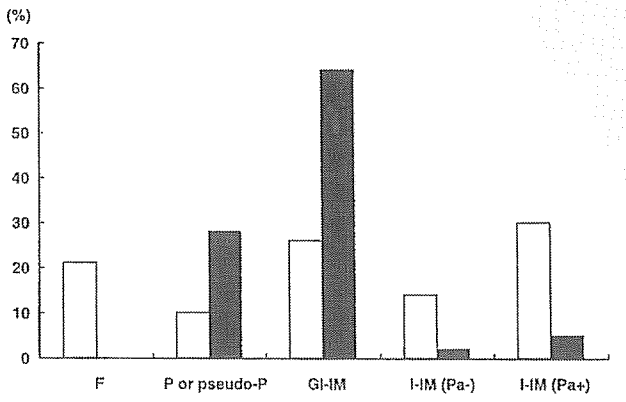

Figure 4 Incidence (%) of phenotypically classified glands in the (□) fundic and (■) pyloric regions. Distributions of GI-IM, I-IM-Pa(–), and I-IM-Pa(+) in these regions are significantly different ($P < 0.0001$). IM, intestinal metaplasia; F, fundic glandular duct; pseudo-P, pseudo pyloric glandular 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.

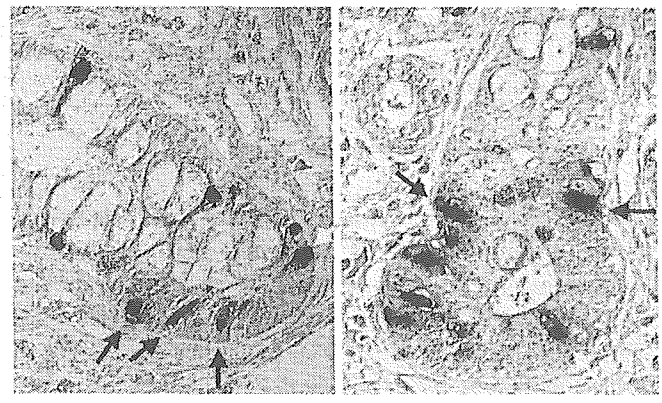

Figure 5 Two examples of double staining of gastrin and glucagon-like peptide (GLP)-1 in the gastric and intestinal mixed phenotype intestinal metaplasia (GI-IM). A mixture of gastrin- and GLP-1-positive endocrine cells are seen not only at the glandular level but also at the cellular level. Gastrin-positive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