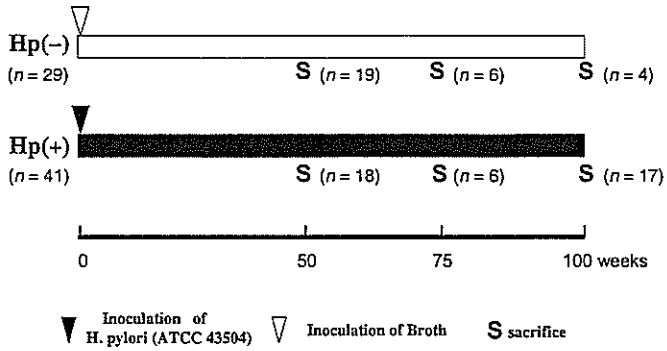

Fig. 1. Experimental design. Hp, *Helicobacter pylori*.

glands was carried out for the pyloric region and jejunum. After death, successful H. pylori infection was confirmed by remarkable elevation of serum IgG titers and/or histological inflammatory change in all H. pylori-infected MG (data not shown).

Each tissue sample was fixed in 95% ethanol plus 1% acetic acid, sectioned at 4 μM, and stained with hematoxylin and eosin for histochemical examination.[18,19]

Immunohistochemistry. Immunohistochemical staining was carried out with the polyclonal antibodies listed in Table 1.[14] The precise procedures for immunohistochemical techniques were as described previously.[14,15,18–22] Briefly, 4 μm-thick consecutive sections were deparaffinized and hydrated through a graded series of ethanol. After inhibition of endogenous peroxidase activity by immersion in 3% H_2O_2/methanol solution, sections were incubated with primary antibodies, washed thoroughly in phosphate-buffered saline (PBS), then incubated with biotinylated secondary antibody followed by the avidin-biotinylated horseradish peroxidase complex (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA, USA). Finally, immune complexes were visualized by incubation with 0.01% H_2O_2 and 0.05% 3.3′-diaminobenzidine tetrachloride (DAB). Nuclear counterstaining was accomplished with Mayer's hematoxylin. Two independent investigators (YT and TT) judged the histology and immunohistochemical staining of each marker.

Classification of glandular ducts in the stomachs of MG. The endocrine cells in each glandular duct were identified as cells with chromogranin A (CgA) cytoplasmic expression. Gastrin is a marker of gastric endocrine cells, whereas GIP is a typical intestinal endocrine cell marker (Table 1).[14]

Regarding the phenotypes of glandular ducts with reference to mucous cell differentiation, we used Alcian blue–periodic acid–Schiff staining (AB-PAS) for identifying gastric surface mucous cells with mucin stained red and goblet cells stained blue (Table 1).[21,23] Non-neoplastic glandular ducts in the stomach were divided histologically and phenotypically into three types: gastric phenotypic glandular (G-type gland), gastric-and-intestinal-mixed phenotypic glandular (GI-type gland), and intestinal phenotypic glandular (I-type gland) ducts.[15,20]

Multiple staining with AB-PAS, gastrin and GIP. Multiple staining with AB-PAS, gastrin and GIP was achieved as follows. After staining with AB-PAS, sections were incubated with anti-gastrin antibody, followed by application of the biotin-labeled goat antirabbit IgG and the peroxidase-labeled avidin–biotin complex method. Binding was visualized with DAB. After thorough washing with Tris-buffered saline, incubation was with the anti-GIP antibody, followed by application of alkaline phosphatase-labeled antirabbit IgG, and development with nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) (BCIP/NBT substrate system for immunohistochemistry and in situ hybridization; Dako, Glostrup, Denmark) using the indirect immuno-alkaline phosphatase method. With this multiple staining, gastrin-positive and GIP-positive cells were stained brown and dark purple, respectively. Glands were classified by AB-PAS staining into G-type, GI-type and I-type glands.

Gland isolation. Gland isolation was carried out as described previously.[20,24] Fresh gastrointestinal tissues were obtained from MG, washed thoroughly in calcium- and magnesium-free PBS to remove the luminal contents and cut into 2–4-cm squares. These were then incubated in calcium- and magnesium-free Hanks' balanced salt solution containing 30 mM ethylenediaminetetraacetate (pH 7.0) and shaken for 15–20 min at 37°C. The gastrointestinal epithelium was then shaven off using the back of a knife, and harvested. The isolated glands were fixed in 70% ethanol and stored at −20°C until RNA extraction.

Sequencing of CgA, gastrin and GIP cDNA in MG. Total RNA from MG stomach and jejunum mucosae were extracted using ISOGEN (Nippon Gene, Toyama, Japan).[20] First strand cDNA was synthesized basically as described previously[25] using random primers with the Thermoscript RT-PCR System (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. To obtain partial cDNA sequences for CgA in MG, the most homologous regions were selected by comparison of the rat (DNA Bank of Japan [DDBJ]/European Molecular Biology Laboratory [EMBL]/GenBank accession number, NM_021655) and mouse (NM_007693) sequences (Table 2). To determine gastrin cDNA sequences, those of rat (NM_012849) and mouse (NM_010257) were compared for selection of one primer pair (Table 2). Regarding GIP, the most homologous regions were selected by comparison with the rat (X66724) and mouse (NM_008119) sequences (Table 2). After successful RT-PCR amplification using cDNA from gerbil stomach and jejunum as a template, sequencing was carried out using the BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster, CA, USA) utilizing ABI Prism 3100 (Applied Biosystems) according to the manufacturer's instructions.

Real-time RT-PCR. The oligo(dT)-primed cDNA was synthesized as described previously, using the Thermoscript RT-PCR System.[20,25] Relative quantitative real-time RT-PCR of CgA, gastrin and GIP was carried out using the LightCycler system (Roche Diagnostics, Mannheim, Germany) with a SYBR Green PCR Kit (Qiagen, Hilden, Germany).[20] The primer sequences of each marker are shown in Table 2. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; DDBJ/EMBL/GenBank accession number AB040445) was used as an internal control. Relative quantification was carried out as established earlier using an internal control, without the necessity for external standards.[25] Values are expressed as a percentage of the CgA-positive and gastrin-positive cells in the pyloric region, and of GIP-positive

Table 1. Phenotypic markers for gastrointestinal endocrine and mucous cells

Cell types	Tissue specificity	Marker	Source of antibody
Endocrine	Ubiquitous	Chromogranin A	Dako (Glostrup, Denmark)
	Gastric	Gastrin	Yanaihara Institute (Fujinomiya, Japan)
	Intestinal	Gastric inhibitory polypeptide	Yanaihara
Mucous	Gastric	Periodic acid–Schiff[1] staining (mucin stained red)	
	Intestinal	Alcian blue[2] staining (mucin stained blue)	

[1]Periodic acid-schiff (Nakalai Tesque, Kyoto, Japan), [2]Alcian blue (Wako Pure Chemical Industries Ltd, Osaka, Japan)

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Table 2. Primer sequences of chromogranin A (CgA), gastrin and gastric inhibitory polypeptide (GIP) in Mongolian gerbils

Gene	Direction	Sequence	Product length (bp)
CgA	Sense	5′-CAAAAGGGGACACCAAGGTGATGAAGTG-3′	153
	Antisense	5′-TCAGCAGATTCTGGTGTCGCAGGATAGA-3′	
Gastrin	Sense	5′-GGAAGCCCCGCTCCCAGCTACAGGATG-3′	171
	Antisense	5′-TCCGTGGCCTCTGCTTCTTGGACAGGTC-3′	
GIP	Sense	5′-AGTGATTACAGCATCGCCATGGACAA-3′	243
	Antisense	5′-CCAGGCCAGTAGCTCTTGAATCAGAAGG-3′	
GAPDH	Sense	5′-AACGGCACAGTCAAGGCTGAGAACG-3′	118
	Antisense	5′-CAACATACTCGGCACCGGCATCG-3′	

GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

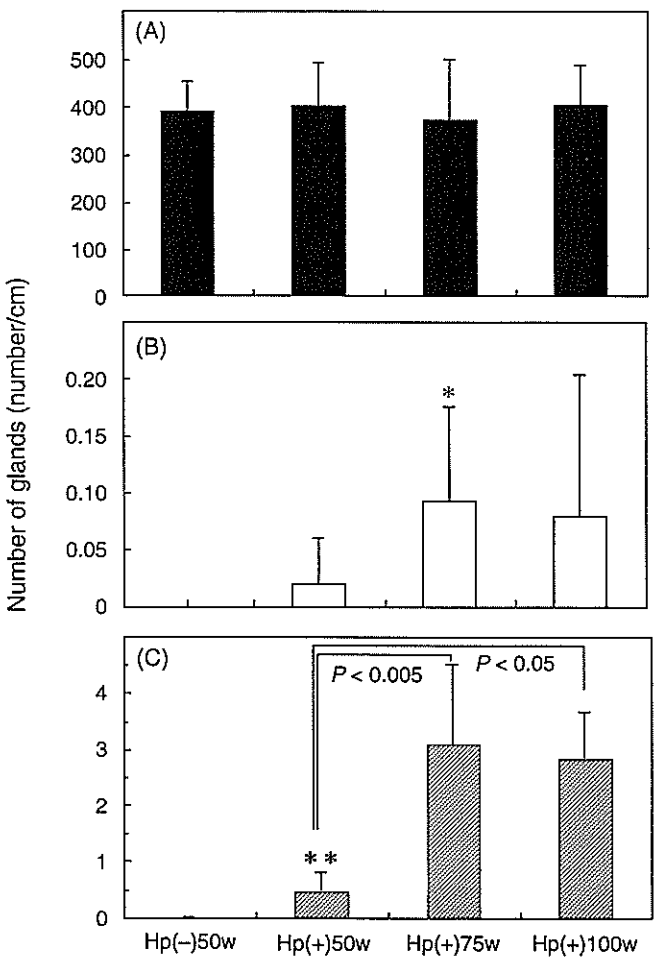

Fig. 2. The average numbers (mean ± SD) of (A) glandular (G)-type, (B) gastric and intestinal mixed (GI)-type and (C) intestinal (I)-type glandular ducts in Hp(−)-50-week ($n = 8$), Hp(+)-50-week ($n = 10$), Hp(+)-75-week ($n = 6$), and Hp(+)-100-week ($n = 9$) cases. *$P < 0.05$, **$P < 0.005$ vs the average Hp(−)-50-week number.

cells in the duodenum (both set as 100%). 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 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).

Statistical analysis. The data were analyzed between groups using the Mann–Whitney U-test. The Kruskal–Wallis test was applied to establish the significance of differences among G-, GI- and I-type glands with reference to gastrin and GIP expression among the Hp(+)-50-week, Hp(+)-75-week and Hp(+)-100-week groups. P-values < 0.05 were considered statistically significant.

Results

Progression of intestinal metaplasia: average numbers of G-, GI- and I-type glands in *H. pylori*-infected MG. Fig. 2 shows the average numbers of G-, GI- and I-type glands per 1 cm of the glandular stomach mucosa (number/cm) in the Hp(−)-50-week (n = 8), Hp(+)-50-week (n = 10), Hp(+)-75-week (n = 6) and Hp(+)-100-week (n = 9) cases. The average numbers of G-type glands were 387.8 ± 62.8 (mean ± SD), 398.5 ± 91.3, 368.8 ± 128.9 and 401.2 ± 83.9, respectively. The average numbers of GI-type glands were 0 ± 0, 0.018 ± 0.039, 0.092 ± 0.083 and 0.079 ± 0.125, respectively, whereas those of I-type glands were 0 ± 0, 0.47 ± 0.32, 3.09 ± 1.40 and 2.83 ± 0.82, respectively. Thus, no GI- or I-type glands were observed in Hp(−)-50-week animals as controls.

The number of I-type glands in Hp(+)-50-week, Hp(+)-75-week and Hp(+)-100-week stomachs was increased significantly compared with the Hp(−)-50-week case ($P < 0.005$). In the Hp(+) groups, the average number of I-type glands increased significantly from week 50 to week 75, and then did not change at week 100. Regarding the GI-type glands, there was a significant difference between Hp(−)-50-week and Hp(+)-75-week ($P < 0.05$).

Immunolocalization of CgA, gastrin and GIP in the normal alimentary tract of MG. Expression of CgA, gastrin and GIP in the normal gastrointestinal tract was estimated by immunohistochemistry in Hp(−)-50-week animals (n = 5) (Fig. 3). The average numbers of CgA-positive cells per 1 cm of the mucosa (number/cm) in fundic regions, pyloric regions, duodenum, small intestines and large intestines were 124.1 ± 19.3 (mean ± SD), 106.7 ± 38.6, 30.7 ± 14.9, 16.9 ± 5.2 and 44.3 ± 25.2, respectively. The expression of CgA decreased gradually from the fundic region to the small intestine and increased from the small to large intestine (Fig. 4A). The average numbers of gastrin-positive cells in fundic regions, pyloric regions, duodenum, small intestines and large intestines were 1.1 ± 1.3, 99.8 ± 39.9, 2.6 ± 3.4, 0 ± 0 and 0 ± 0, respectively. No gastrin expression was detected in the small and large intestines. The average numbers of GIP-positive cells in fundic regions, pyloric regions, duodenum, small intestines and large intestines were 0.6 ± 0.6, 1.1 ± 0.5, 25.2 ± 12.1, 22.3 ± 7.5 and 0.9 ± 0.2, respectively. GIP expression was observed frequently in the small but not the large intestine.

Immunohistochemical analysis of CgA, gastrin and GIP in the pyloric regions of *H. pylori*-infected MG stomachs. For immunohistochemical analyses of CgA, gastrin and GIP, stomach samples of eight Hp(−)-50-week, six Hp(−)-75-week, four Hp(−)-100-week, ten Hp(+)-50-week, six Hp(+)-75-week and nine Hp(+)-100-week

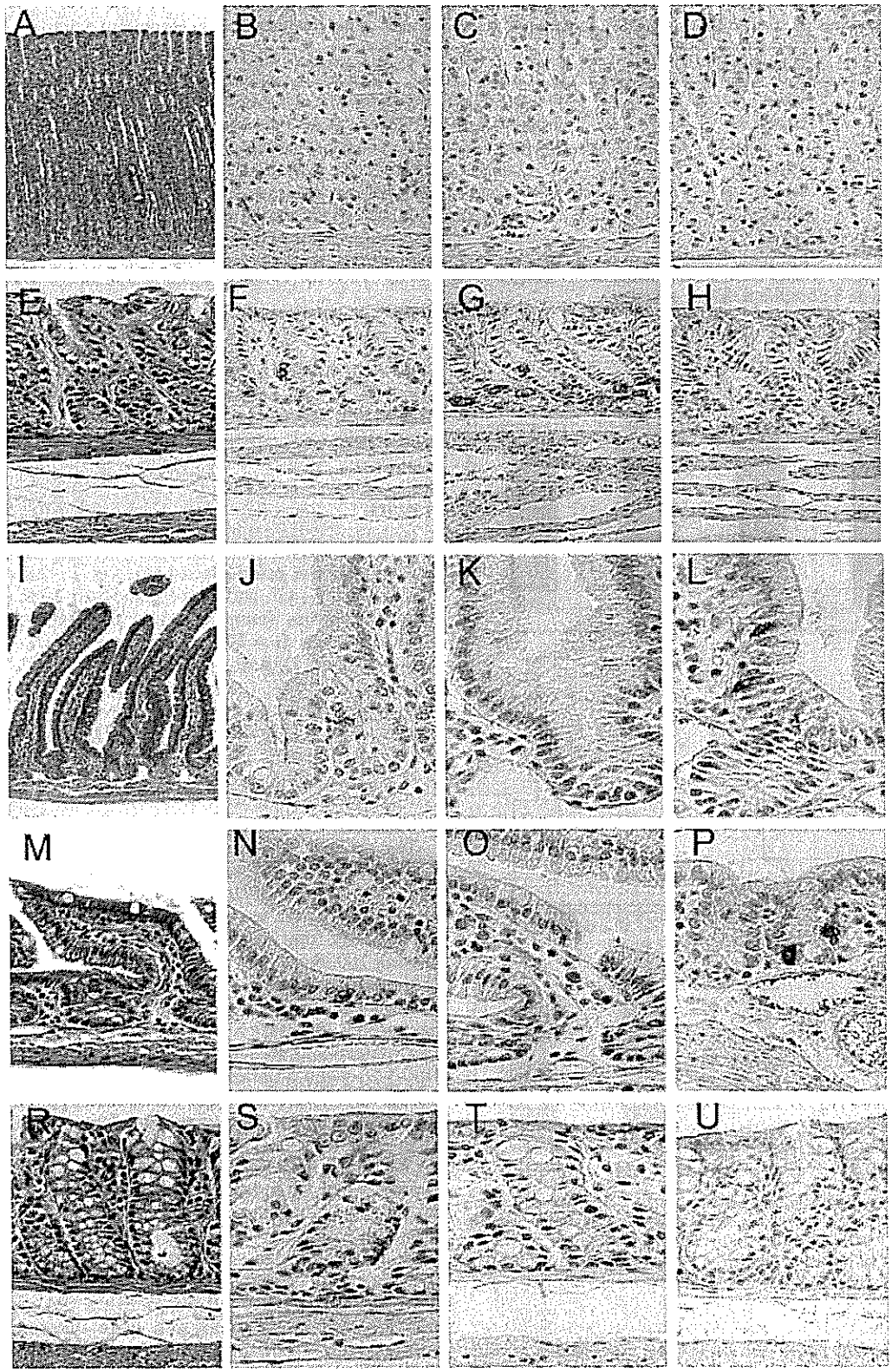

Fig. 3. Immunohistochemical staining of chromogranin A (CgA), gastrin and gastric inhibitory polypeptide (GIP) in (A–D) normal fundic and (E–H) pyloric regions, (I–L) duodenum, (M–P) small intestines and (R–U) large intestines of Mongolian gerbils. Expression of CgA was observed in the bottom of (B) normal fundic, (F) pyloric, (J) duodenal, (N) small intestinal and (S) colonic glandular ducts. (C,D) No gastrin or GIP was observed in the fundic glands. In the pyloric glands, (G) expression of gastrin was detected clearly, but (H) no GIP expression was observed. In the duodenum and small intestine, (L,P) GIP expression was detected, but (K,O) no gastrin expression was observed. Neither (T) gastrin nor (U) GIP were detected in the large intestine. Original magnification: (A) ×100; (B–U) ×400.

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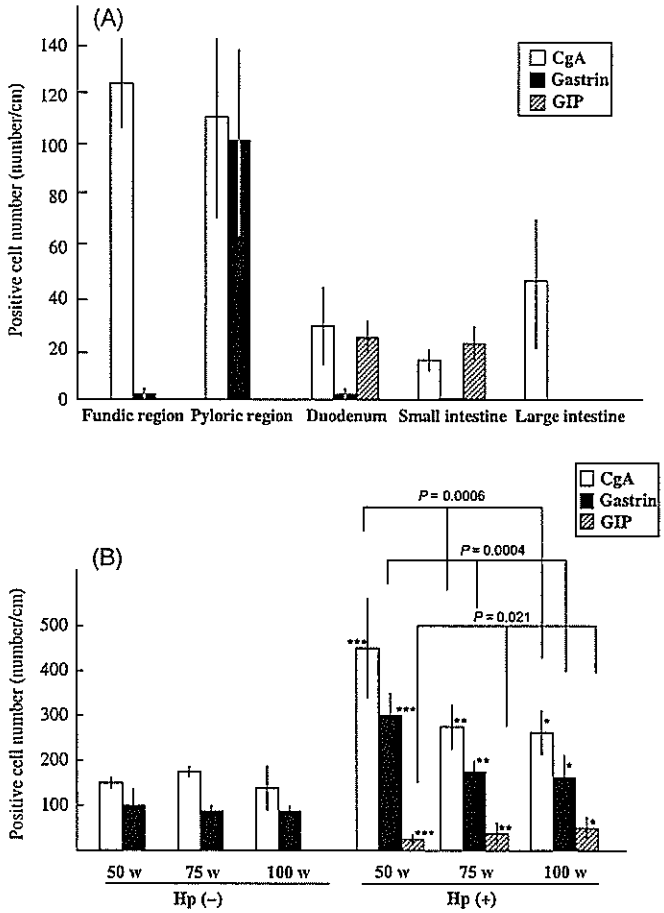

Fig. 4. Immunohistochemical analysis of chromogranin A (CgA)-, gastrin- and gastric inhibitory polypeptide (GIP)-positive cells. (A) Normal gastrointestinal tract. (B) *Helicobacter pylori*-infected Mongolian gerbils. *$P < 0.01$, **$P < 0.005$, ***$P < 0.0005$ compared with each control group.

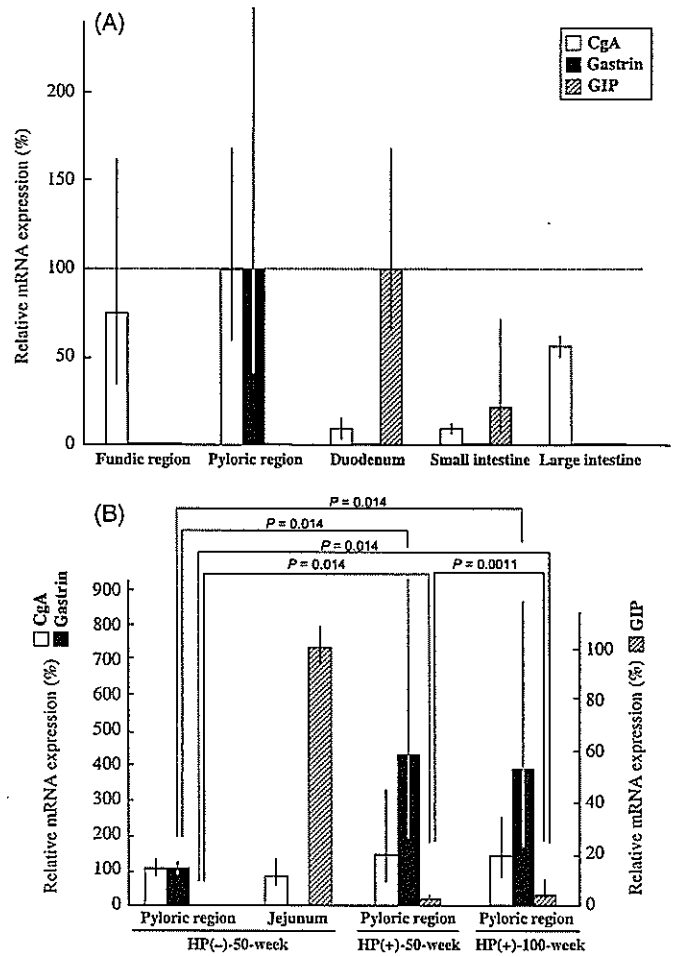

Fig. 5. Real-time reverse transcription–polymerase chain reaction analysis of chromogranin A (CgA), gastrin and gastric inhibitory polypeptide (GIP) in the pyloric region of Mongolian gerbils. (A) Normal alimentary tract. (B) Isolated pyloric glands from *Helicobacter pylori*-infected stomachs.

animals were used (Fig. 4B). The average numbers of CgA-positive cells per 1 cm of the pyloric region (number/cm) were 156.7 ± 6.4, 175.7 ± 2.5, 136.3 ± 60.4, 446.1 ± 104.2, 271.9 ± 175.4 and 250.0 ± 60.0, respectively. There was no significant variation in the number of CgA-positive cells in the controls at the three time points. Values were elevated in each H. pylori-infected group, but decreased gradually from Hp(+)-50-week through Hp(+)-75-week to Hp(+)-100-week ($P = 0.0006$). The average number of gastrin-positive cells in Hp(–)-50-week, Hp(–)-75-week, Hp(–)-100-week, Hp(+)-50-week, Hp(+)-75-week and Hp(+)-100-week were 96.1 ± 46.7, 83.7 ± 8.6, 92.7 ± 11.5, 295.1 ± 54.1, 175.4 ± 24.5 and 162.7 ± 44.7, respectively. The average number of GIP-positive cells was 1.9 ± 1.4, 2.1 ± 0.2, 2.8 ± 1.8, 20.0 ± 5.0, 35.5 ± 15.0 and 40.4 ± 24.3, respectively.

Determination of partial nucleotide sequences of CgA, gastrin and GIP. Partial nucleotide sequences of neuroendocrine markers in the MG were determined and deposited at the DNA Data Bank of Japan (DDBJ) (http://www.ddbj.nig.ac.jp/Welcome-e.html). DDBJ/EMBL/GenBank The accession numbers for CgA, gastrin and GIP are AB253527, AB253528 and AB253529, respectively.

Expression of CgA, gastrin and GIP mRNA in the normal alimentary tract. The expression of CgA, gastrin and GIP mRNA in the normal gastrointestinal tract was also estimated by real-time RT-PCR in Hp(–)-50-week animals (n = 3) (Fig. 5A), values being expressed as a percentage of those in the pyloric mucosa of stomach for CgA and gastrin, and relative to that in the duodenum for GIP. The average relative expression levels of CgA in fundic regions, pyloric regions, duodenum, small intestines and large intestines were 74.9% (35.05% [mean – SD]–160.11% [mean + SD]), 100% (59.87–167.02%), 10.25% (6.29–16.69%), 9.39% (7.62–11.56%) and 57.04% (51.50–63.17%), respectively. The corresponding figures for gastrin were 1.69% (0.45–6.34%), 100% (39.36–254.08%), 1.12% (0.15–8.34%), 0.0036% (0.00032–0.041%) and 0.015% (0.000024–8.76%), and for GIP were 0.00019% (0.000059–0.00064%), 1.30% (0.46–3.67%), 100% (61.69–162.11%), 22.85% (7.00–74.60%) and 0.0088% (0.0014–0.057%). In line with the immunohistochemical and real-time RT-PCR findings, gastrin and GIP were used as gastric and intestinal endocrine cell markers, respectively, in MG.

Alteration of CgA, gastrin and GIP mRNA expression in isolated pyloric glands from *H. pylori*-infected MG. We used gland isolation to avoid contamination of epithelial cell elements with stromal or inflammatory cells. Isolated glands were obtained from the pyloric regions of Hp(–)-50-week (n = 3), Hp(+)-50-week (n = 8) and Hp(+)-100-week (n = 8) animals. Examples were also collected as controls of GIP mRNA expression from Hp(–)-50-week jejunums (n = 3). Values are expressed as the percentage of those in isolated glands obtained from the pyloric region for

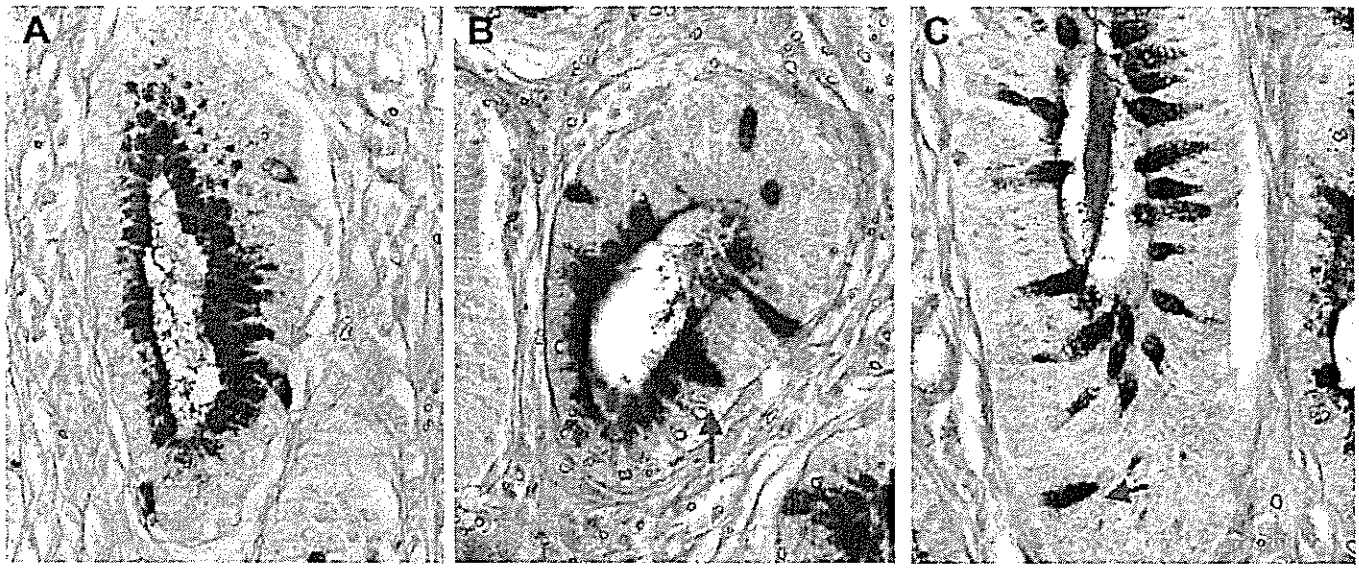

Fig. 6. Expression of gastrin and gastric inhibitory polypeptide (GIP) in (A) glandular (G)-type, (B) gastric and intestinal mixed (GI)-type and (C) intestinal (I)-type glands. (A) In the G-type glands, gastrin-positive cells were identified by brown cytoplasmic staining (red arrow), but no GIP expression was observed. (B) In the GI-type glands, gastrin- and GIP-positive cells were identified by brown (red arrow) and dark purple (blue arrow) cytoplasmic staining. (C) In the I-type glands, GIP expression was detected by dark purple staining (blue arrow), but no gastrin expression was apparent. Original magnification: (A–C) ×500.

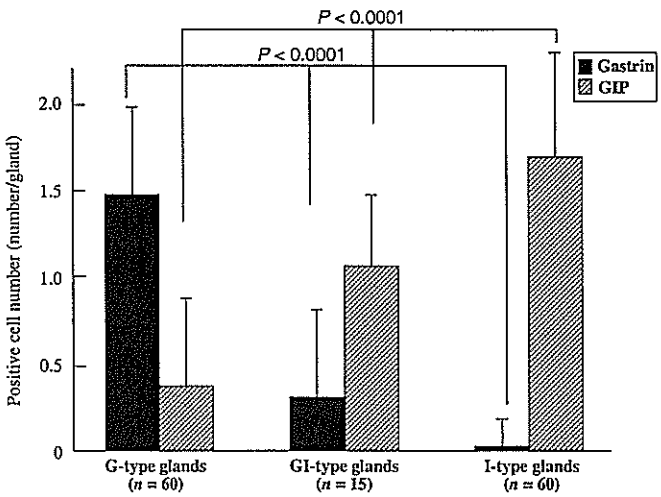

Fig. 7. Expression of gastrin and gastric inhibitory polypeptide (GIP) in glandular (G)-type, gastric and intestinal mixed (GI)-type and intestinal (I)-type glands. The number of gastrin-positive cells decreased gradually from G-type through GI-type to I-type glands ($P < 0.0001$), correlating inversely with the number of GIP-positive cells ($P < 0.0001$).

CgA and gastrin and in the jejunum for GIP (Fig. 5B). Average relative expression levels for CgA mRNA were 100% (81.50–122.70%), 80.11% (54.32–118.14%), 136.60% (55.78–334.55%) and 147.55% (82.65–263.43%) in the normal Hp(–)-50-week pyloric region and the jejunum, and in the H. pylori-infected pyloric regions of Hp(+)-50-week and Hp(+)-100-week animals, respectively. Those for gastrin mRNA were 100% (89.95–111.17%), 0.21% (0.10–0.44%), 436.46% (205.35–927.65%) and 399.88% (182.48–876.31%), respectively, and for GIP mRNA were 0.00057% (0.000037–0.0086%), 100% (92.68–107.90%), 0.29% (0.14–0.57%) and 3.38% (1.40–8.17%), respectively.

The expression of gastrin mRNA in H. pylori-infected groups (Hp[+]-50-week and Hp[+]-100-week) was increased significantly compared with the Hp(–)-50-week case. However, there was no significant difference in gastrin mRNA expression between Hp(+)-50-week and Hp(+)-100-week groups. The expression of GIP mRNA in H. pylori-infected groups (Hp[+]-50-week and Hp[+]-100-week) was also increased significantly compared with Hp(–)-50-week. In addition, there was a significant difference in GIP between Hp(+)-50-week and Hp(+)-100-week (Fig. 5B). Regarding CgA mRNA expression, there was no statistically significant variation among the groups.

Colocalization of gastrin- and GIP-positive endocrine cells of GI-type glands. The presence of gastrin- and GIP-positive cells was double-immunohistochemically evaluated with AB-PAS staining (Fig. 6) to directly compare the localization of endocrine and mucous G-type and I-type markers. The numbers of gastrin-positive and GIP-positive cells per gland (number/gland) were analyzed in 60 G-type, 15 GI-type and 60 I-type glands of H. pylori-infected groups. The average numbers of gastrin-positive cells were 1.48 ± 0.57, 0.53 ± 0.51 and 0.03 ± 0.18 in G-, GI- and I-type glands, respectively. Those for GIP-positive cells were 0.15 ± 0.36, 1.00 ± 0.53 and 1.68 ± 0.65, respectively (Fig. 7). Gastrin-positive cells decreased gradually from G- through GI-, to I-type glands ($P < 0.0001$), whereas GIP-positive cells were correlated inversely ($P < 0.0001$) (Fig. 7). Coexistence of gastrin- and GIP-positive cells was detected in the same gland in GI-type glands (Fig. 6).

Discussion

To our knowledge, this is the first report of expression of endocrine and mucous cell markers observed periodically in the glandular stomach of H. pylori-infected MG, although several studies have shown that long-term H. pylori colonization produces hyperplasia of gastrin-producing antral G-cells and carcinoid tumors in MG.[7,9,26] In the present study, the immunohistochemical data demonstrated that the numbers of CgA- and gastrin-positive cells in H. pylori-infected groups was increased significantly compared with the non-infected condition, but both demonstrated a gradual decrease over time, despite the lack of any significant variation in CgA or gastrin mRNA expression between Hp(+)-50-week and Hp(+)-100-week. In humans, there have been several

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reports of no significant differences in the number of G-cells and G-cell density in the stomach mucosa between H. pylori-infected and -uninfected healthy volunteers,[27–29] although the number of G-cells was significantly less in patients with both H. pylori infection and duodenal ulcer than in either infected or uninfected controls.[27–30] Kamada et al. reported that G-cell number in H. pylori-associated gastritis mucosa was decreased in comparison with an uninfected case.[30] Diamaline et al. suggested that CgA production in enterochromaffin-like cells of the rat stomach was part of the functional response of these cells to circulating gastrin.[31] We consider that the expression of some factors, including CgA and gastrin, is influenced by the time after H. pylori infection, and further analyses in the MG model may explain the discrepancy with human reports. Regarding the relationship between IM and the change in endocrine cells, several reports demonstrated that G-cells disappeared and I-type endocrine cells conversely appeared in human IM mucosa,[4–6] and that many Glicentin-positive, intestinal phenotype cells were found at the IM gland level,[6,32] in line with our gland isolation findings. However, few GIP-positive cells were observed in non-infected groups. H. pylori infection may trigger intestinalization of the stomach mucosa.[21] We have showed previously that the phenotypes of endocrine cells are associated strongly with those of mucous cells in human IM,[14] and the GI-type glands in the present study had both gastrin- and GIP-positive cells in the H. pylori-infected MG glandular stomach. This evidence supports the concept that all of the different types of mucous and endocrine cells may be generated from a single stem cell.[15]

Regarding intestinalization of the stomach mucosa, changes in the expression of various genes, especially homeobox examples determining cell structures and functions, may be involved. The fetal stomach, which develops from the foregut, displays areas of I-type mucosa with goblet cells and epithelial cells with striated borders in the antrum and cardia.[33] It is important to consider the correlation between expression of phenotypes and organ-specific genes. We earlier showed that Sox2 and Cdx1/2 are gastric- and intestinal-specific transcription factors, respectively.[15,20] In isolated pyloric and intestinal metaplastic glands, the phenotypes of mucous cells were found to be associated strongly with these specific transcription factors. In isolated GI-type glands, Sox2 and Cdx1/2 were both observed, as well as gastric and intestinal mucous cell markers such as MUC5AC, MUC6, MUC2 and villin. Recently, La Rosa et al. demonstrated that Cdx2 may be a sensitive and specific marker of midgut endocrine cells and endocrine tumors.[34] We think that Cdx2 might be important in the regulation of intestinal phenotype endocrine cell markers such as GIP, glicentin and glucagon-like polypeptide-1 because of its localization. Jenny et al. reported previously that neurogenin3, a basic helix-loop-helix transcription factor, is required for endocrine cell fate specification in multipotent intestinal progenitor cells, whereas gastric endocrine development is both neurogenin3 dependent and independent.[35] Thus, specific transcription factors, including Cdx2, might play an important role in the intestinalization of endocrine cells as well as mucous cells, because a phenotypic link was here observed sequentially between mucous and endocrine cells. Moreover, recently, several reports have demonstrated that impaired expression of the stomach morphogenic factor Sonic hedgehog (Shh) by parietal cells and increased expression of the transcriptional activators of intestinal and pancreatic differentiation, namely CDX2 and PDX1, are crucial for the development of stomach atrophy and for intestinal, endocrine and pancreatic transdifferentiation processes.[36,37] Suzuki et al. described that prolonged colonization by H. pylori led to extension of inflammation from the antrum to the corpus of the stomach with downregulation of Shh in gastric epithelial differentiation in the MG model.[38] Again, evaluation of the expression of the specific transcription factors detailed above should be carried out periodically in the H. pylori-infected MG model.

Our present data demonstrate that most glands in the stomach of H. pylori-infected MG were G-type. The numbers of GI- and I-type glands were extremely low in comparison. In the non-infected MG, no GI- or I-type glands were detected. We have demonstrated previously that most stomach cancers present with gastric phenotypic expression in the glandular stomach of H. pylori-infected MG treated with carcinogens.[21] In the rat, Tatematsu et al. reported that pepsinogen 1-altered pyloric glands, which are low in pepsinogen 1, are putative preneoplastic lesions in the glandular stomach.[39–41] In humans, most early-stage gastric cancers consist mainly of G-type cancer cells, irrespective of histological type.[15,42–44] Thus, it is important to note the possibility that precancerous lesions exist in G-type as well as IM glands.[21]

In conclusion, the phenotype of endocrine cells is in line with that of their mucous cell counterparts in the glands of the H. pylori-infected MG stomach, supporting the concept that the development of IM is due to abnormal differentiation of stem cells. For elucidation of stomach carcinogenesis, it is very important to evaluate factors related to H. pylori infection periodically in the MG model.

Acknowledgments

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Review article

Gastric-and-intestinal mixed-type intestinal metaplasia: aberrant expression of transcription factors and stem cell intestinalization

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Abstract

***Helicobacter pylori* plays a causative role in the development of chronic atrophic gastritis, intestinal metaplasia (IM), and stomach cancer. Although IM has long attracted attention as a putative preneoplastic lesion for stomach cancers, its clinicopathologic significance has yet to be clarified in detail. Using gastric and intestinal epithelial cell markers, IM was here divided into two major types: a gastric-and-intestinal (GI) mixed type and a solely intestinal (I) type. In the former, gastric and intestinal phenotypic markers appeared not only at the glandular but also at the cellular level. Furthermore, neuroendocrine cells also showed intestinalization along with their exocrine counterparts. In animal models, GI-type IM was found to appear first, followed by the solely I type. Summarizing these data, it was suggested that IM might be caused by the gradual intestinalization of stem cells from the GI to the I type. The molecular mechanisms of IM include the ectopic expression of CDX1, CDX2, OCT-1, and members of the Erk pathway. Suppression of the expression of gastric transcription factors such as SOX2, genes that are involved in the Sonic hedgehog pathway, and RUNX3, a tumor suppressor gene, could be additional relevant alterations. The expression of PDX1 may also be associated with pseudopyloric gland metaplasia and IM. Detailed analysis of gene regulation may shed light on the molecular bases of gastric lesions, leading to strategies for chemoprevention.**

Key words Gastric-and-intestinal mixed-type intestinal metaplasia · Stem cell · Transcription factor

Introduction

Since the discovery of *Helicobacter pylori* by Warren and Marshal [1] in Australia, it has been well established that this microorganism plays important roles in the development of chronic gastritis, intestinal metaplasia (IM), and stomach cancers, including malignant lymphomas [2–7]. In 1994, the World Health Organization (WHO)/International Agency for Research on Cancer (IARC) categorized *H. pylori* as a group 1 "definite carcinogen" [8]. IM has been extensively studied as a putative preneoplastic lesion in the human stomach, due to its strong association with stomach cancer development [9–18]. Although controversy exists as to its real significance [19,20], IM is considered by some to be a precancerous lesion for so-called intestinal adenocarcinomas of Lauren's classification [21]. This type comprises well- and moderately differentiated adenocarcinomas, irrespective of the presence of the intestinal properties. Clearly, the pathogenesis of IM, as well as its molecular background, needs to be detailed for the elucidation of the actual relation between IM and stomach cancer [22].

Stem cells in the gastrointestinal mucosa

To investigate the cellular origin of tissues, mosaicism of cellular genetic markers is often used. One approach is to use chimeric animals, produced experimentally by the amalgamation of cells from allelically different strains. Recently, numerous histological markers have also been applied for the analysis of mosaicism in chimeric mice. Antibodies strictly recognizing C3H strain-specific antigens (CSAs) [23] enable the immunohistochemical discrimination of C3H cells in histological sections of chimeric mouse tissues. In normal gastric and intestinal mucosa of chimeric mice, each gland is composed entirely of CSA-positive or -negative cells, and no mixed glands are found, indicating that each individual gland in the adult mouse is derived from a single progenitor cell. Surface mucous cells (foveolar epithelial cells), mucous neck cells, parietal cells, and chief cells in the fundic glands thus all arise from the same cell. Similarly, surface mucous cells and pyloric gland cells arise from a single progenitor cell [24–26].

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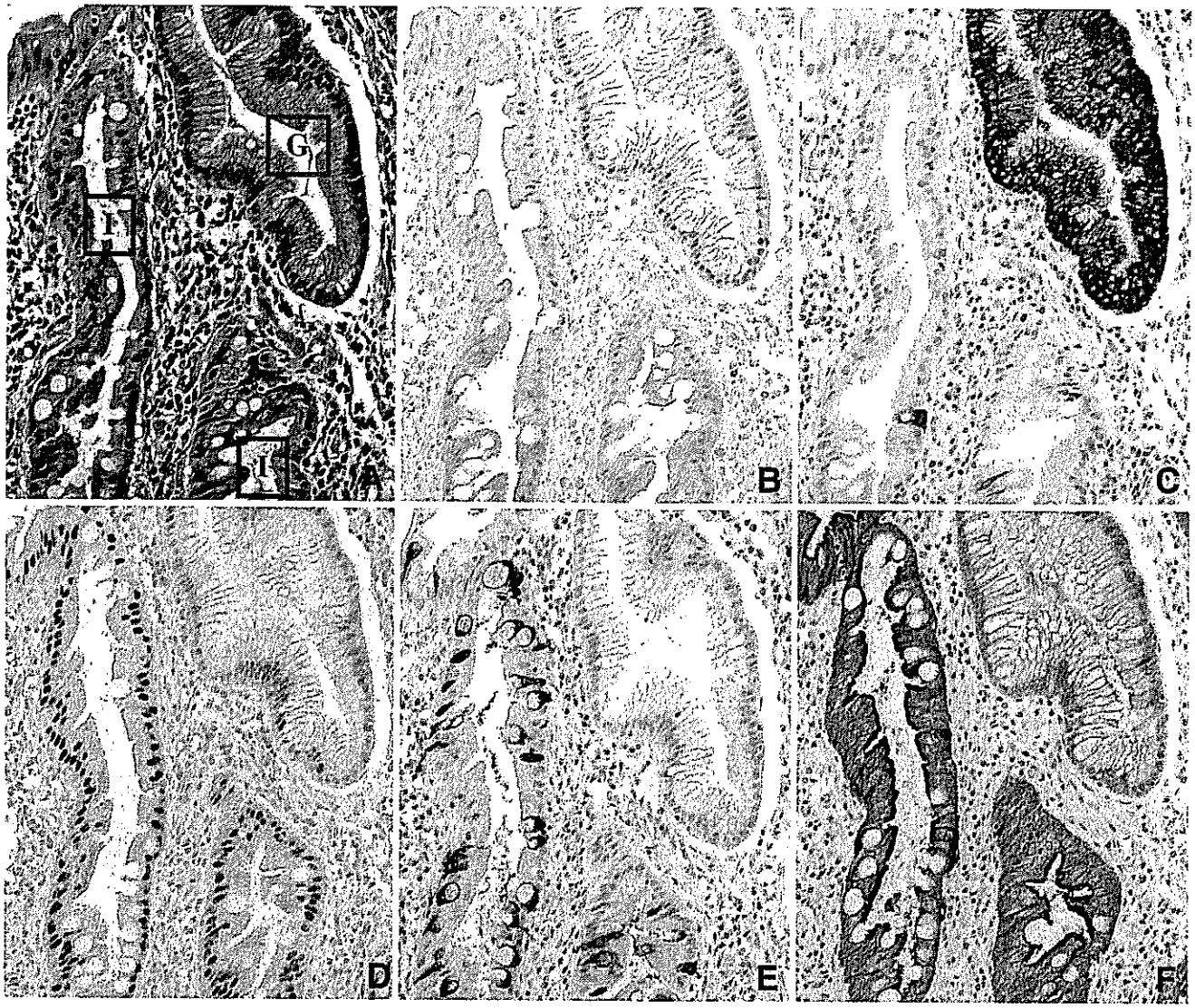

Fig. 1A–F. Immunohistochemical staining of normal human gastric mucosa and intestinal metaplasia. *G*, Pyloric gland with a gastric phenotype expressing Sox2 and MUC5AC; *I*, intestinal metaplastic gland harboring goblet cells producing Cdx2, MUC2, and villin. **A** H&E staining; **B–F** immunohistochemistry; for Sox2 (**B**), MUC5AC (**C**), Cdx2 (**D**), MUC2 (**E**), and villin (**F**). Binding was visualized with 3,3′-diaminobenzidine (DAB), and counterstaining was done with light green SF yellowish (**B**) or hematoxylin (**C–F**). ×200

Another approach to showing cellular phenotypic mosaicism in females utilizes the random inactivation of one X chromosome [27–29]. The human androgen receptor gene locus (HUMARA) has been used to assess methylation, and about half of intestinal metaplastic glands were revealed to be heterotypic (comprised of cells with differing allelic methylation), while the remainder were homotypic (cell populations with the same allelic methylation). Mouse models have also been used to show heterogeneity within a single gland/crypt, utilizing the Dlb-1 locus, which determines the expression of the binding site for the lectin *Dolichos biflorus* agglutinin (DBA) in the intestinal epithelium, in C57BL/6J x SWR F1 mice [30]. Mouse models have also shown X-linked glucose-6-phosphate dehydrogenase (G6PD) activity in C3H/Heston mice [31]. When a carcinogen, ethylnitrosourea, was administered to the C3H/Hester mice, loss of G6PD activity appeared in one side of a colonic crypt [31]. These results led to the hypothesis of the existence of four to six stem cells in one crypt [32].

The discrepancy between the concept of a single progenitor stem cell and the hypothesis of four to six stem cells in one crypt could be derived from confusion regarding the terminology used for "stem cells". The single stem cell in glands/crypts hypothesized from the

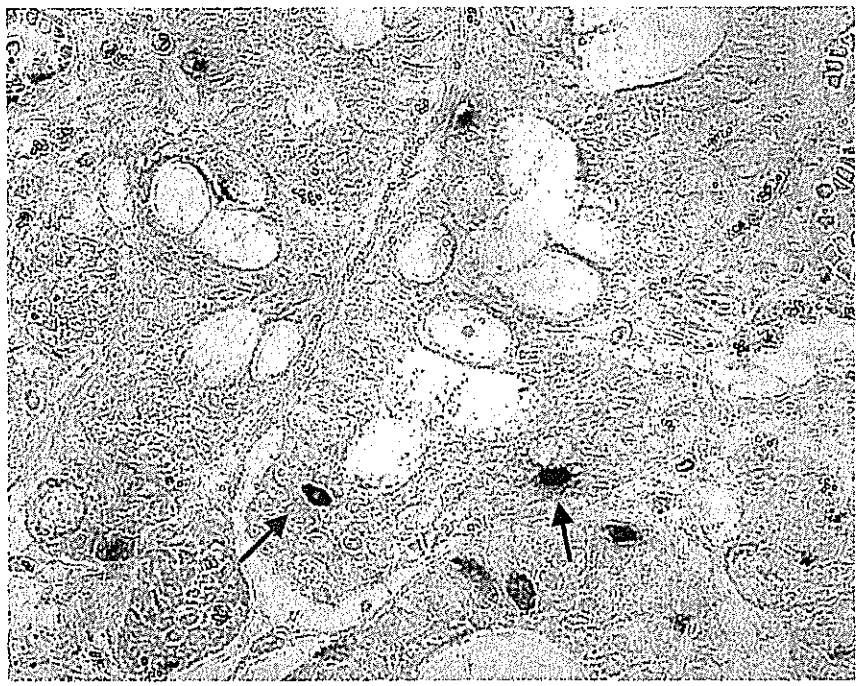

Fig. 2. An example of double staining of gastrin and glucagon-like peptide (GLP)-1 in gastric-and-intestinal mixed phenotype intestinal metaplasia (GI-IM). A mixture of gastrin- and GLP-1-positive endocrine cells is apparent in the same gland. Gastrin-positive cells (*blue arrow*) and GLP-1-positive cells (*red arrow*) are indicated. ×200

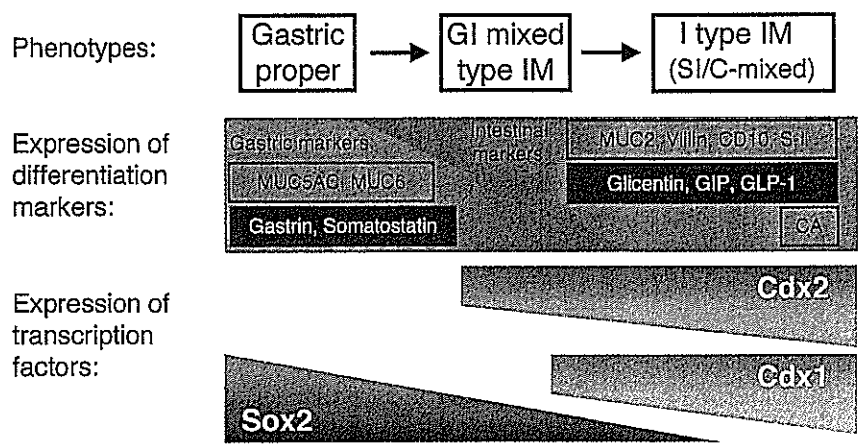

Fig. 3. Schematic view of progression of intestinal metaplasia (*IM*). Gastric mucosa changes to gastric-and-intestinal (*GI*) mixed-type IM, and then progresses to Solely intestinal (*I*)-type IM. Within the I type, small-intestinal (*SI*) type cells can colocalize with G phenotype cells, whereas colonic (*C*) type cells appear in complete I-type IM. Sox2, along with gastric markers, is decreased, and Cdx and intestinal markers emerge ectopically during the progression of IM. *S-I*, sucrase-isomaltase; *CA*, carbonic anhydrase 1; *GIP*, gastric inhibitory peptide

chimeric mouse data may be a "master stem cell" commanding the whole gland, whereas the four to six stem cells in one crypt, indicated by the latter experiments [31,32], could be "committed stem cells", obeying the master stem cell in producing their progeny.

Gastric and intestinal epithelial cell markers

Mucins in the alimentary tract can be divided into two main classes: class III mucins in mucous neck cells, pyloric gland cells, and Brunner's gland cells; and class II mucins, in surface mucous cells, goblet cells, and the surface coat of intestinal absorptive cells, as assessed utilizing paradoxical concanavalin A staining [33]. With more recent developments in mucin histochemistry and immunohistochemistry, intestinal metaplastic cells can now be clearly classified, by the analysis of phenotypic expression, into a gastric epithelial cell type (G type), resembling pyloric gland cells and surface mucous cells, and an intestinal epithelial cell type (I type), resembling goblet and intestinal absorptive cells. Gastric mucosa consists of foveolar cells in the upper two-thirds and pyloric gland cells in the lower one-third. Concerning gastric phenotypic markers, the surface mucous-cell type contains galactose oxidase-Schiff (GOS) and sialidase-GOS reactive mucin, positive for mucin core protein (MUC), MUC5AC. Cells of the pyloric gland cell type

contain class III mucin, colocalized with MUC6, and show pepsinogen reactivity. Intestinal metaplastic mucosa consists of absorptive cells with a brush border, goblet cells packed with clear rounded vacuoles containing mucin, and, sometimes, Paneth cells, harboring eosinophilic granules in their cytoplasm, which usually appear at the bottom of the glands. Regarding intestinal markers, the goblet-cell type contains mucin that is GOS-negative and sialidase-GOS reactive, possessing sialyl-Tn antigen and MUC2 core protein. Cells of the intestinal absorptive type demonstrate sucrase and intestinal-type alkaline phosphatase activity, harboring CD10 as a surface marker, and the structural protein villin. Cells of Paneth-cell type are reactive with anti-defensin antibodies [34–41] (Fig. 1, Table 1).

Classification of intestinal metaplasia (IM)

The present widely applied classification of IM, into complete and incomplete types, was first proposed by Matsukura and colleagues [10] and Kawachi and colleagues [42]. Classification based upon mucin secretion patterns as well as morphology has also allowed division into a small-intestine type and a colonic type [43,44]. Jass and Filipe [45] described three grades of IM (types I, II, and III) on the basis of morphology and classical mucin staining, using periodic acid-Schiff, Alcian blue (AB), and high iron diamine (HID) methods. Type I corresponds to the complete type and types II and III to the incomplete type. While these classifications are generally accepted, they are based only upon the intestinal properties and do not take into account the gastric properties that are still preserved in association.

We have therefore proposed a new classification, based upon the cell differentiation status, using both gastric and intestinal cell phenotypic markers [39]. With this classification, IM is divided into two major types; a gastric-and-intestinal (GI) mixed type, and a solely intestinal (I) type. To confirm this histological classification, stomach mucosa was subjected to gland isolation and classification of individual glands into gastric (G), GI mixed, and I types according to the preservation of pyloric cells and the appearance of goblet cells, as revealed with Alcian blue and paradoxical concanavalin A staining. The G type preserves the pyloric cells without the emergence of goblet cells. In the I type, intestinal metaplastic glands consist of goblet and intestinal absorptive cells, with or without Paneth cells. In the GI mixed-type, on the other hand, gastric phenotype cells are found together with intestinal phenotype cells in various-combinations. All of the subtypes of GI mixed-type IM and a subtype of the I type without Paneth cells belong to the incomplete IM category, while the I type with Paneth cells corresponds to complete-type IM. In many cases of the GI mixed type, atrophied pyloric glands are present under the intestinalized glands.

Mixtures of gastric and intestinal phenotypes occur at the cellular as well as the glandular level. Intestinal metaplastic glands are easily found on hematoxylin and eosin (H&E) staining, by the presence of goblet cells and brush border lining the apical side of the epithelium. Goblet cells have been confirmed to show an intestinal phenotype, as shown with MUC2 immunostaining, which is not present in gastric epithelium. The brush border is positive for villin, as shown in normal intestinal epithelium. However, gastric mucin sometimes remains in both goblet and absorptive cells, as revealed by MUC5AC immunohistochemistry, with villin expression being weaker in MUC5AC-positive cells as compared to those without MUC5AC expression. Thus, IM subtypes should not be considered as independent entities, but, rather, as a sequence of pathological states with a gradual change from gastric to intestinal character. GI mixed-type IM may be composed of mixtures of cells with various degrees of intestinal phenotypic shift, rather than being just a random mixture of gastric- and intestinal-type cells. This allows us to introduce the notion that IM may be due to abnormal stem cell differentiation, but with some stem cells still obeying certain orders.

It is believed that stem cells (multipotent progenitor cells) are present in the proliferative cell zone in the isthmus region of gastric glands, giving rise to all the various cell types by differentiation, so that, consequently, gastric glands are monoclonal in the adult stage [46,47]. In the environment of a normal gastric gland, cells derived from stem cells undergo complex bipolar migration from the isthmus, either upward or downward. In the pyloric mucosa, surface mucous cells move upward, while pyloric gland cells migrate downward [48]. In the crypts of the small intestine, on the other hand, stem cells would be expected to be present in the proliferative cell zone at the bottom of the crypts. In the normal intestinal gland, cells that will become absorptive and goblet cells move up, and only those differentiating into Paneth cells migrate lower from the proliferative cell zone. In GI mixed-type IM, gastric surface mucous cells, intestinal absorptive cells, and goblet cells are found in the glandular portions above the proliferative zone, while pyloric gland cells and Paneth cells are found in the lower glandular portions, below the proliferative zone [40]. GI mixed-type IM may be the consequence of the abnormal differentiation of stem cells that can produce both gastric- and intestinal-type cells, with the normal cell migration pattern preserved. Because epithelial cell differentiation and the migration of gastric glands are thought to be closely linked, it is not clear why only the former is disturbed.

Table 1. Differentiation markers for the human gastrointestinal tract

Differentiation markers	Gastric				
	Foveolar cell	Pyloric cell	Fundic mucous neck cell	Fundic chief cell	Fundic parietal cell
Structural proteins					
Functional proteins					Proton pump alpha subunit[e] Proton pump beta subunit[e]
Enzymes		Pepsinogen II[e]		Pepsinogen I[f] Pepsinogen II	
Mucin core proteins	MUC5AC[a]	MUC6[a]	MUC6[a]		
Mucins	HGM[a] SH9[k] GOS staining PAS staining	PCS			
Neuroendocrine hormone					
Transcription factors	Sox2[j]	Pdx1[n]		RUNX3[o]	

HGM, human gastric mucin; GOS, galactose oxidase Schiff staining; PAS, periodic acid Schiff staining; PCS, paradoxical concanavalin A staining; I-ALP, intestinal alkaline phosphatase; CA1, carbonic anhydrase 1; SIMA, small intestinal mucious antigen; S-GOS, sialidase GOS; GIP, gastric inhibitory peptide; GLP-1, glucagon-like peptide 1

Sources of available antibodies

[a] Novocastra (Newcastle upon Tyne, UK)
[b] Transduction Laboratory (Lexington, KY, USA)
[c] Medical and Biological Laboratories (MBL) (Nagoya, Japan)
[d] Dr. E. M. Porter, University of California, Los Angeles
[e] Biogenesis (Poole, England, UK)
[f] Dr. M. Ichinose, Wakayama Medical College
[g] Dr. T. Irimura, Tokyo University
[h] DAKO (Glostrup, Denmark)
[i] Dr. K. Hirano, Gifu Pharmaceutical University
[j] Chemicon (Temecula, CA, USA)
[k] Dr. Imai, Nara Medical College
[l] Dr. S. Hakomori (Tokyo University)
[m] Yanaihara Institute (Fujinomiya, Shizuoka, Japan)
[n] Dr. Y. Yuasa, Tokyo Medical and Dental University
[o] Dr. Y. Ito, Institute of Molecular and Cell Biology, Singapore
[p] Biogenex (San Ramon, CA, USA)

	Intestinal			
Neuroendocrine cell	Absorptive cell	Goblet cell	Paneth cell	Neuroendocrine cell
	CD10[a] Villin[b]			
			Defensin 5[d]	
	Sucrase-isomaltase[g] I-ALP[i] CA1[j]		Lysozyme[b]	
		MUC2[a]		
		SIMA[a] TKH2[l] Sialyl-Tn antigen[l] 91.1H[g] S-GOS staining Alcian blue staining		
Chromogranin A[h] Gastrin[l] Somatostatin[h]				Chromogranin A[hl] Glicentin[l] GIP[l] GLP-1[l]
	Cdx1 Cdx2[p]	Cdx1 Cdx2	Cdx1 Cdx2	

Sequential analysis using animal models

Experimentally, the shift from GI mixed-type IM to I-type IM can be observed in sequential observations in animal models. The occurrence of IM in rats gradually increases with time after X-ray irradiation; the number of GI mixed-type IMs is relatively high at 2–4 weeks, becoming lower thereafter. On the other hand, the number of I-type IMs is extremely low at 2 weeks, and then increases with time. These observations suggest that the phenotype of IM sequentially changes from the GI mixed-type to the I type [49].

H. pylori infection in Mongolian gerbils causes IM in their glandular stomachs [50]. Twenty-five weeks after inoculation with *H. pylori*, the glandular stomach epithelium becomes hyperplastic, and heterotopic proliferating glands (HPGs) penetrate the muscularis mucosae. Fifty weeks after infection, intestinal metaplastic cells appear among gastric epithelial cells, including goblet cells possessing Alcian blue-positive mucins and/or absorptive cells with a striated brush border, so that the lesions are characterized as GI-mixed-type IM. At 75 weeks, HPGs with gastric phenotype decrease and most animals possess solely I-type HPGs. Paneth cells appear by 100 weeks.

The *N*-methyl-*N*-nitrosourea-induced mouse stomach carcinogenesis model also provides support for the conclusion that intestinalization of the stomach epithelium occurs in late stages, as assessed by monitoring intestinal alkaline phosphatase expression [51].

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

Gastrointestinal glands possess neuroendocrine cells, usually in their bottom regions, among the mucous and absorptive cells. Gastrin-positive endocrine cells are predominantly detected in the normal pyloric mucosa, with some detected in the duodenal mucosa. Somatostatin-positive cells are also mainly detected in the normal pyloric mucosa, with some detected in the fundic and duodenal mucosae. Glicentin, gastric inhibitory peptide (GIP)-, and glucagon-like peptide 1 (GLP-1)-positive endocrine cells are detected exclusively in the duodenum, small intestine, and colon, but not in the normal gastric mucosa. Therefore, gastrin and somatostatin could be gastric-predominant endocrine cell markers, whereas glicentin, GIP, and GLP-1 characterize the intestinal phenotype.

In GI mixed-type IM glands, both gastric and intestinal endocrine markers have been found to be present in endocrine cells, correlating with the phenotypic expression of the glands. Thus, in I-type IM glands harboring only intestinal mucous cell markers, endocrine cells demonstrate only intestinal endocrine peptides. However, double immunostaining for gastrin and GLP-1 has revealed the existence of both gastric and intestinal endocrine cells in the same glands of the GI-mixed-IM type. Furthermore, at the single cell level, quite a few glands harbored endocrine cells that were positive for both gastrin and GLP-1 (Fig. 2).

All of the different types of mucous, absorptive, and endocrine cells in normal as well as intestinal metaplastic glands may be derived from a single progenitor cell. In the light of the clonal findings with C3H/HeN↔BALB/c chimeric mice, we consider that the alteration from gastric to intestinal metaplastic glands must be controlled at the stem-cell level.

Expression of transcription factors in intestinal metaplasia (IM)

CDX homeobox gene family

Caudal-type homeobox (*Cdx*) 1 and *Cdx2* are mammalian members of the caudal-related homeobox gene family [52]. In the adult mouse, and in humans, expression is strictly confined to the gut, from the duodenum to the rectum. Silberg et al. [53] reported the presence of Cdx1 protein in intestinal metaplastic lesions of the human stomach, and Mizoshita et al. [54] demonstrated the expression of *Cdx1* and *Cdx2* in both the small and large intestine, and in intestinal metaplastic mucosa of the human stomach. Eda et al. [55] found that the expression of *Cdx2* preceded that of *Cdx1* during the progression of IM. Satoh et al. [56] described *Cdx2* expression in the gastric epithelium of *H. pylori*-infected patients, with or without obvious IM. *Cdx2* plays an important role in the intestine-specific expression of carbonic anhydrase 1 [57]. Furthermore, it stimulates the intestine-specific expression of sucrase-isomaltase [58], lactase-phlorizin hydrolase [59], and guanylyl cyclase C [60]. More recently, *Cdx2* has been revealed to induce the expression of MUC2 mucin in goblet cells [61]. Cdx1 has been reported to appear in intestinal metaplastic glands, as described by Silberg et al. [53]. Its expression is strong in regenerating epithelial foci, but not in quiescent sterilized crypts after irradiation-induced damage [62], and recent analyses have shown that Cdx1 is a direct transcriptional target of the Wnt β-catenin signaling pathway during mouse gut development [63] and that Cdx1 is stimulated by oncogenic β-catenin in human colon cancer cells [64]. Dietary factors may be involved in the suppression of *Cdx2* via its promoter methylation [65] (Fig. 3).

Sox gene family

To analyze the shift from a gastric to an intestinal phenotype, one should also focus on gastric transcription factors, including the *Sox* gene family [66], which consists of ten subgroups, divided according to Sry-like high-mobility group (HMG) box homology. The *Sox* genes in group B1, including *Sox1*, *Sox2*, and *Sox3*, are important for gut development in mice [67]. In-situ analysis of the chicken *cSox2* gene demonstrated localized expression in the embryonic endoderm, with transcripts appearing before the commencement of morphogenesis, and cytodifferentiation in the rostral gut epithelium from the pharynx to the stomach. The caudal limit of *cSox2* expression coincides with that of the region competent for proventricular differentiation and with the rostral limit of the domain of *CdxA* [68]. In the human digestive tract, *Sox2* expression is found in stomach epithelium, including the fundic and pyloric mucosae, but is very low in the intestine, as observed in the chicken. However, in IM, *Sox2* transcripts begin to decrease and gradually disappear as IM progresses from the GI-mixed-type to the I type, with *Sox2* showing an inverse correlation with *Cdx1* and *Cdx2*. *Sox2* may regulate the expression of gastric differentiation markers, including MUC5AC, as suggested in the chicken system [69]. The expression patterns of *Sox2* and *Cdx1/Cdx2* are inversely related, and down-regulation of *Sox2* could thus be an important mechanism in IM, in addition to the ectopic expression of *Cdx1/Cdx2* [70]. Specificity of the expression pattern of these transcription factors also persists in stomach cancers [71,72] (Fig. 3).

PDX1

Pancreatic-duodenal homeobox 1 (*PDX1*), a *ParaHox* gene which contributes to the genesis and development of the pancreas, duodenum, and antrum, has been found to be frequently expressed in pseudopyloric glands and IM. MUC6 is more abundant than MUC5AC in pseudopyloric glands, while higher levels of MUC5AC than MUC6 are evident in IM. In carcinomas, *PDX1* expression is closely associated with MUC6, whereas no link is apparent between *PDX1* and MUC5AC reactivity. Thus, *PDX1* may play an important role in the development of pseudopyloric glands and subsequent IM [73,74].

OCT-1

OCT-1 is a member of the POU homeodemain family of transcription factors [75]. This protein recognizes the canonical octamer motif (ATGCAAAT) and is implicated in the activation of the mouse Cdx2 promoter in pancreatic and intestinal cell lines. OCT-1 is expressed in chronic gastritis, particularly when it is adjacent to IM, and it is also expressed in 87% of IM foci. Furthermore, 74% of gastric carcinomas in one series were found to be positive for OCT-1, and a strong association was observed between OCT-1 expression and an intestinal-type phenotype. OCT-1 is able to bind to the CDX2 promoter, although transactivation of CDX2 has not been demonstrated [76].

Sonic hedgehog (Shh) pathway

High levels of *Shh* are expressed in the fundic glands of the stomach in the normal gastrointestinal tract, but *Shh* expression is lost in IM of the human stomach [77], resulting in a glandular phenotype of intestinal transformation and overgrowth. Hedgehog-related transcription factors, Gli2 and Gli3, may be involved in *Shh* signaling. While disruption of Gli2 (the principal factor mediating the activator function of Shh), leads to minimal changes in glandular development in the mutant mouse, knockout of Gli3, functioning as a repressor of the Hedgehog signal, causes a striking phenotype of glandular expansion and intestinal transformation. A reduction in apoptotic events was seen in the stomachs of all Gli3 mutants, without affecting proliferation [78]. In humans, impaired expression of the gastric morphogenic factor *Shh* by parietal cells, and the increased expression of transcriptional activators of intestinal and pancreatic differentiation; namely, *CDX2* and *PDX1*, seem to be crucial for the development of gastric atrophy and for intestinal, endocrine, and pancreatic transdifferentiation processes [74].

Erk pathway

The increased expression of villin is one of the earliest changes seen in *H. pylori* infection [70]. These bacteria have been found to stimulate the villin promoter in a human gastric adenocarcinoma cell line (AGS) via activation of the Erk pathway, where Elk-1 and the serum response factor (SRF) are downstream transcriptional targets. Inducible binding of Elk-1 and the SRF to the proximal promoter of villin after 3 and 24 h of treatment with *H. pylori* suggests that these bacteria alone are sufficient to initiate a cascade of signaling events responsible for villin expression.

Runt-related transcription factor gene 3 (RUNX3)

The RUNX family of transcription factors plays pivotal roles during normal development and in neoplasias [79], and *RUNX3* is reported to be a tumor suppressor gene for stomach cancer [80]. The loss of *RUNX3* expression due to aberrant methylation of its CpG island (evident in gastric cancer cell lines) suggests that this factor is a

target for epigenetic gene silencing in gastric carcinogenesis. *RUNX3* methylation has also been found in mucosa with chronic gastritis or IM [81]. Immunohistochemistry disclosed RUNX3 protein in most chief cells and a few gastrin-containing G cells in normal mucosa, but not in IM or carcinoma cells [82]. Furthermore, in vitro studies have shown that gastric epithelial cells can differentiate into intestinal-type cells, probably due to the expression of *Cdx2*, when the function of *Runx3* is impaired in *Runx3*-knockout mice [83].

Expression of small-intestinal and colonic phenotypes in complete intestinal metaplasia (IM)

Jass and Filipe [45] described three grades of IM, in terms of small-intestinal sialomucin and colonic sulfomucin expression, shown by high-iron diamine alcian blue (HID-AB) staining. Type I glands have no mucins in columnar cells, but feature goblet cells. Type II glands have blue-stained columnar cells possessing sialomucins, while type III glands harbor brown-stained columnar cells producing sulfomucins, with type II and III glands characterized by slight distortion. To discriminate small-intestinal and colonic differentiation in IM, molecular markers, including sucrase and carbonic anhydrase 1 (CA1) could be utilized in comparison with MUC5AC mucin core protein. CA1 expression is detectable in the cytoplasm of colon epithelial cells (especially on the luminal side of the colonic mucosa), but not in the jejunum. Sucrase, on the other hand, is present on the luminal surfaces of mature small-intestinal absorptive cells, but not in the colon. In IM, gastric MUC5AC expression is higher in CA1-negative mucous cells of GI-mixed-type IM glands, compared with CA1-positive I-type IM, in line with levels of MUC5AC mRNA. In contrast, the expression of sucrase is more strongly detected on the luminal surfaces of CA1-positive IM gland cells than in CA1-negative IM glands. MUC2, villin, and *Cdx2* expression is observed in intestinal metaplastic cells, irrespective of CA1 expression. The number of glands with CA1 expression is higher in type I complete IM compared to types II and III incomplete IM. Furthermore, there appear to be no differences between types II and III in terms of CA1 expression, and no correlation of colonic sulfomucin expression. In short, the expression of gastric and colonic markers may be regulated in a different manner, although both can be colocalized with small-intestinal markers [84] (Fig. 3).

Conclusion

Atrophic gastritis and IM of the stomach mucosa are generally considered to be precancerous lesions, and chronic *H. pylori* infection is one of the most important factors in their development. However, *H. pylori* strains show a wide variety at the genome level, especially regarding the *cag* and *vac* genes, and this variation may underlie the observed large differences in stomach cancer incidence and mortality around the world, including the "Asian paradox" and "African enigma". In addition to bacterial factors, polymorphisms in host genes (for example, for cytokines that modulate inflammatory responses) are believed to exert synergistic effects. For the prevention of detrimental changes in the stomach mucosa, it is necessary to elucidate the pathogenetic mechanisms of mucosal atrophy and IM due to *H. pylori* infection.

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Severity of gastritis determines glandular stomach carcinogenesis in *Helicobacter pylori*-infected Mongolian gerbils

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Helicobacter pylori (H. pylori) infection causes chronic gastritis and is also related to gastric carcinoma. The present study focused on severity of *H. pylori*-induced gastritis as a determinant of carcinogenesis. Seven-week-old male Mongolian gerbils were inoculated with *H. pylori* at experimental weeks 0, 12, or 18, then given *N*-methyl-*N*-nitorosourea (MNU) from weeks 20–40. At week 70, stomachs were then excised for histological examination 70, 58, or 52 weeks after *H. pylori* inoculation, respectively (Groups A, B, and C for long-, middle-, and short-term). The respective incidences of glandular stomach adenocarcinomas were 65.0% (13/20), 20.0% (2/10), and 23.0% (3/13) ($P < 0.05$). Higher scores of infiltration of inflammatory cells, hyperplasia, intestinal metaplasia and mucosal bromodeoxyuridine (BrdU) labeling index in antrum and corpus mucosa, were seen in group A than B or C ($P < 0.05$) and serum anti-*H. pylori* IgG titer and gastrin levels were also significantly higher, along with mRNA levels for mucosal interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), cyclooxygenase-2 (COX-2), and inducible nitric oxide synthase (iNOS). The results demonstrated the term and severity of *H. pylori* infection to play important roles in gastric carcinogenesis, with essential involvement of chronic inflammation, especially increased rates of cell proliferation, in *H. pylori*-associated carcinogenesis. (*Cancer Sci* 2007)

A large body of evidence points to a close relationship between Helicobacter pylori (H. pylori) infection and the development of chronic gastritis, peptic ulcers and gastric carcinomas.[1,2] Recently the concept that inflammation is a critical component of tumor progression has received a great deal of attention. Many cancers arise from sites of infection, chronic irritation and inflammation,[3] and H. pylori can induce cytokine release from epithelial cells which leads to recruitment of inflammatory cells. Reactive oxygen species generated by neutrophils causes lipid peroxidation, as well as protein and DNA oxidation. In addition, persistent inflammation has an impact on cellular turnover.[4,5]

It is clear that not all H. pylori infections are linked to gastric cancer, since approximately 50% of the world population is infected, and only a very small minority of infected subjects suffer from an associated neoplasm. The reasons for this phenomenon are unknown although different mechanisms have been proposed.[6,7] In humans, studies have shown that H. pylori gastritis is associated with increased risk, and this decreases following cure of bacterial infection.[8] The gastritis model in Mongolian gerbils has advantages in that the human pathogen H. pylori is used and the consequent chronic gastritis, intestinal metaplasia, and gastric cancer closely mimic those in humans.[9,10] Previous studies showed that H. pylori infection induces chronic active gastritis, expression of various cytokines and regenerative epithelial cell responses in gerbils.[11,12] However, the level of gastric mucosal injury has received only limited attention in studies of H. pylori-induced gastritis. For this study, we therefore designed an experiment with different timing and periods of infection in animals of the same ages. Our hypothesis was that early inoculation and long-term colonization of H. pylori would result in more severe chronic active gastritis, and therefore a greater yield of gastric carcinomas.

Materials and Methods

Animal and *H. pylori* inoculation. Seven-week-old male specific-pathogen-free Mongolian gerbils (Meriones unguiculatus; MGS/Sea) were purchased from Seac Yoshitomi, Ltd. (Fukuoka, Japan) and maintained in an air-conditioned biohazard room with free access to a commercial rodent diet (Oriental CRF-1; Oriental Yeast Co., Tokyo, Japan) and water ad libitum. H. pylori ATCC 43504 [American Type Culture Collection (ATCC), Manassas, VA, USA] were grown in Brucella broth supplemented with 7% fetal calf serum at 37°C under microaerophilic conditions for 48 h. Animals were inoculated with 0.8 mL of broth culture containing 1×10^8 colony-forming units (cfu) of H. pylori by gastric intubation three times at 48-h intervals. The animals were fasted for 24 h before the first inoculation. All experiments and procedures carried out on the animals were approved by the Animal Care Committee of Aichi Cancer Center Research Institute.

Chemical. N-methyl-N-nitrosourea (MNU) (Sigma Chemical Co., St Louis, MO) was dissolved in distilled water at a concentration of 10 p.p.m. (solution was freshly prepared three times per week) for administration in light-shielded bottles as drinking water ad libitum.

Experimental protocol. The animals were divided into six H. pylori -inoculated and two control groups (Fig. 1). H. pylori were inoculated for groups A and E at 0 weeks, groups B and F at 12 weeks, and groups C and G at 18 weeks, representative of early long-term, middle, and short-term infection, respectively. Since the early long-term Group A was predicated that it might be of poorer survival rate at the end of the experiment, more animals were added into Group A than Groups B and C. Groups D and H received Brucella broth without H. pylori. At 20 weeks, Groups A, B, C, and D were given MNU. Before the MNU administration, subgroups of animals in groups A, B, and C (a1, b1, and c1) were sacrificed at 20, 12, and 2 weeks post-infection, respectively. For these gerbils, 5′-bromo-2′-deoxyuridine (BrdU) at a dose of 100 mg/kg, was injected intraperitoneally, 60 min before the sacrifice. All animals were subjected to deep ether anesthesia after 24 h fasting, laparotomized, and exsanguinated from the inferior vena cava, after which their stomachs were excised.

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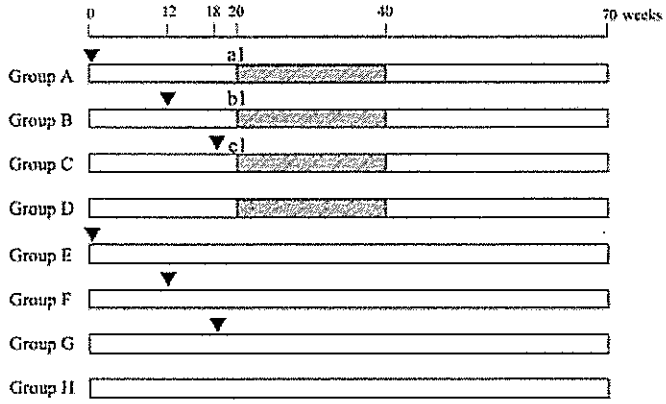

Fig. 1. Experimental design. Animals, 7-week-old male Mongolian gerbils, ▼, *H. pylori* (i.g.), ■, *N*-methyl-*N*-nitorosourea in drinking water at the concentration of 10 p.p.m.

Table 1. PCR primers used for real-time quantitative RT-PCR analysis

Gene	Primer	Product size (bp)
GAPDH	F: 5′-AACGGCACAGTCAAGGCTGAGAACG-3′ R: 5′-CAACATACTCGGCACCGGCATCG-3′	118
IL-1β	F: 5′-TGACTTCACCTTGGAATCCGTCTCT-3′ R: 5′-GGCAACAAGGGAGCTCCATCAC-3′	91
TNF-α	F: 5′-GCTGCCCCCACCTCGTGCTC-3′ R: 5′-CTTGATGGCAGACAGGAGGCTGACC-3′	89
COX-2	F: 5′-GCCGTCGAGTTGAAAGCCCTCTACA-3′ R: 5′-CCCCGAAGATGGCGTCTGGAC-3′	97
iNOS	F: 5′-GCATGACCTTGGTGTTTGGGTGCC-3′ R: 5′-GCAGCCTGTGTGAACCTGGTGAAGC-3′	110

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL-1β, interleukin-1β; TNF-α, tumor necrosis factor-α; COX-2, cyclooxygenase-2; iNOS, inducible nitric oxide synthase; F, forward; R, reverse.

Histopathological evaluation. Multiple 4 μm-thick histologic sections were obtained from routinely processed 4% paraformaldehyde-fixed and paraffin-embedded tissues. Sections were stained with hemotoxylin and eosin (H&E) or with Alcian blue (pH 2.5)-periodic acid Schiff (AB-PAS) for detection of mucin-containing cells. The glandular mucosa of the antrum and corpus was examined histologically for inflammatory and epithelial changes. Active chronic gastritis was characterized by infiltration of neutrophils, mononuclear cells, hyperplasia, and intestinal metaplasia. The degree of change was graded on a scale from 0 to 3, [0 (normal), 1 (mild), 2 (moderate), and 3 (marked)], based on the Updated Sydney System.[13] Epithelial cell proliferation was assessed by BrdU labeling, visualized using a mouse monoclonal anti-BrdU antibody (1:50, Dako, Glostrup, Denmark) as described previously.[14] The numbers of BrdU-labeled cells in the gastric mucus of antrum and corpus were counted under a microscope with a ×40 objective lens, and indices were determined as the mean percentages of positive cells among totals of more than 1000 cells. Mucosal thickness was assessed using NIH Image version J1.272 (National Institutes of Health, USA).

Analysis of cytokines by real time quantitative PCR. Total RNA was extracted from the glandular stomach mucosa at the border between the antrum and corpus using an RNA extraction kit (Isogen, Nippon Gene, Tokyo, Japan). After DNase treatment, first strand cDNAs were synthesized using the Thermoscript RT-PCR System (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Relative quantitative PCR of interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), cyclooxygenase-2 (COX-2), and inducible nitric oxide synthase (iNOS),[15] was performed with the LightCycler system (Roche Diagnostics, Mannheim, Germany), using the gerbil-specific glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene as an internal control.[16] PCR was performed basically as described earlier using a QuantiTect SYBR Green PCR (QIAGEN) kit with optimal Mg^{2+} concentration at 2.5 mM.[17] 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 quantification was performed as previously established using an internal control without the necessity for external standards.[17] The expression levels of cytokine mRNAs were expressed relative to 1.0 in the control group (group H).

Serology. Serum samples were used to measure the titers of anti-H. pylori IgG antibodies by enzyme-linked immunosorbent assay, and serum gastrin levels were measured at 70 weeks using radioimmunoassay. The antibody titers were expressed using an arbitrary index, and values greater than the cut-off of 1.5 were considered to be positive for H. pylori infection.[18]

Statistical analyses. Results for the gastritis scores, BrdU labeling indices, mRNA expression levels, and antibody and gastrin levels are given as means ± SE. The Fisher's exact test and the Bonferroni multiple-comparison test were performed to establish the significance of differences with the cut-off at $P < 0.05$.

Results

Histopathology. The survival rates in all groups were >85%, with no differences among groups. All gastric mucosal specimens from control gerbils demonstrated a normal histomorphology. Histological findings for gastric mucosal specimens from H. pylori-infected gerbils are summarized in Table 2. At 20 weeks, the early/long-term H. pylori-infected gerbils (group a1) showed greater lymphoplasmocytic infiltration and submucosal lymphoid follicle formation than the middle and short-term H. pylori-infected groups in the antral mucosa (Table 2; Fig. 2a,b). At 70 weeks, there was a change over time in topography of the gastritis from predominantly antral gastritis to pangastritis in H. pylori-infected gerbils. Lesions of gastric mucosa were more marked in the long-term infection groups than in the middle and short-term infected groups (Table 2; Fig. 2c,d). Heterotopic proliferative glands (HPGs) were observed in all H. pylori-infected gerbils at 77 weeks,[19] limited to the antrum and the junctional region between the antrum and the body (Fig. 2c).

Incidence of adenocarcinomas. Data for adenocarcinomas are summarized in Table 3. Both well differentiated (Fig. 3a) and signet ring-cell carcinomas (Fig. 3b) were found, mainly located in the antrum or at the border between the antrum and the corpus. Whereas 13 of 20 (65%) in the long-term H. pylori + MNU group (group A) had adenocarcinomas in the glandular stomach, this was the case for only two of 11 (20.0%) in the middle-term H. pylori + MNU group (group B), and three of 13 (23.1%) in the late short-term H. pylori + MNU group (group C). The difference was statistically significant. In the MNU-alone group (group D), H. pylori-alone groups (groups E, F, and G), and controls (group H), no tumors developed in the glandular stomach.

BrdU labeling indices for epithelial cells. At 20 weeks, antrum BrdU labeling indices in group a1 were significantly greater than in groups b1 and c1 ($P < 0.01$), with no significant increase in the corpus. At 70 weeks, both antrum and corpus BrdU labeling indices in the long-term infection groups (A and E) were significantly elevated as compared to middle and short infection groups (B and F, and C and F) ($P < 0.05$) (Table 1).

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