Immunohistochemical analysis of Cdx2 in gastric cancers with submucosal invasion

Of the 60 early GC with submucosal invasion, 48 and 12 were judged to be Cdx2-positive and -negative, independent of the histological type (Table 2). In the GI- and I-type GC, the areas of the Cdx2-positive nuclear staining were in perfect accord with those of intestinal phenotypic expression. The G- or N-type GC with <10% of cancerous areas with intestinal phenotypic expression also had nuclear staining of Cdx2 in their intestinal phenotypic cancer cells, without exception. However, four differentiated and nine undifferentiated cancers were judged to be Cdx2-positive in 22 G-type intramucosal cancers. In these cases, Cdx2 nuclear staining was detected in >10% of cancerous areas, not only where intestinal phenotypic expression was evident but also in tissue displaying only gastric markers. Similarly, one differentiated and two undifferentiated cancers were judged to be Cdx2-positive in six N-type intramucosal cancers, in which Cdx2 staining was also detected outside the cancerous area showing intestinal phenotype. The rates for Cdx2-positive cases in differentiated and undifferentiated lesions were 90.0% and 70.0%, respectively, the difference not being significant ($P = 0.053$). Expression of Cdx2 was not associated with lymph node metastasis (data not shown).

Analysis of the phenotypic expression in non-neoplastic glands bordering microcarcinomas

The clinicopathologic findings, phenotype and Cdx2 expression for six intramucosal microcarcinomas, which measured less than 3.0 mm in the largest dimension of the microcarcinoma, and six glandular ducts in the immediately adjacent non-neoplastic surrounding mucosa, are shown in Table 3. The phenotypes and Cdx2 expression of the microcarcinoma did not correlate with those of non-neoplastic surrounding mucosa.

DISCUSSION

Our present data provide clear evidence that most early GC are Cdx2-positive with immunohistochemical staining evident in 102 (78.5%) of 130 lesions. This appeared independent of the degree of invasion with 54 (77.1%) of 70 intramucosal cancers and 48 (80.0%) of 60 cancers with submucosal invasion, being positive. Several reports have pointed to a tumor-suppressor potential of Cdx2 in human colorectal tumorigenesis.[30,41–43] Bai *et al.* have demonstrated evidence that Cdx2 upregulates transcription of p21/WAF1/CIP1 which plays critical roles in differentiation and tumor suppression using HT-29 colon carcinoma cells.[44] Yuasa has also reported that Cdx2 promotes intestinal differentiation by upregulating p21/WAF1/CIP1 as a cyclin-dependent kinase inhibitor leading to cell-cycle arrest.[45] In GC, we and others have previously shown that Cdx2-positive tumors have a significantly better outcome than negative lesions suggesting that Cdx2 might suppress the invasion of cancer cells.[10,32] Taking into account these previous reports and our present data, we consider that Cdx2 might suppress the expansion of cancerous areas in the early stage of gastric carcinogenesis accompanied by intestinalization of GC cells.

We have previously shown experimentally induced adenocarcinomas in the rat glandular stomach to consist mainly of gastric epithelial phenotypic cancer cells, with intestinal epithelial phenotypic cancer cells appearing later in larger tumors. This suggests a phenotypic shift from gastric to intestinal phenotypic expression during the course of tumor progression.[14,15,21,22] We and others have also reported that this phenotypic shift occurs in accordance with increasing depth of invasion in human signet ring cell carcinomas and with progression in human differentiated GC.[5,16,24,25] Tatematsu *et al.* demonstrated the incidence of the cancer cells with intestinal phenotypic expression in early differentiated cases to be higher than that in undifferentiated counterparts.[23] A shift from gastric to intestinal phenotypic expression was observed mucin histochemically and immunohistochemically

Table 3 Relations between six microintramucosal carcinomas and surrounding mucosa

Age (years)	Sex	Location	Size (mm)	Depth	Histological type	Phenotype	Cdx2	Surrounding mucosa
65	Male	L	0.9	m	Differentiated	N type	Positive	Six GI-IMs
40	Male	M	1.7	m	Differentiated	I type	Positive	Three pyloric glandular ducts Three GI-IMs
60	Male	L	1.9	m	Differentiated	GI type	Positive	Six pyloric glandular ducts
61	Male	M	2.3	m	Undifferentiated	G type	Negative	Three I-IMs Two pyloric glandular ducts One GI-IM
60	Male	L	2.4	m	Differentiated	GI type	Positive	Three pyloric glandular ducts Three GI-IMs
54	Male	L	2.8	m	Undifferentiated	G type	Negative	Five pyloric glandular ducts One GI-IM

M and L, middle and lower one-third of the stomach; m, intramucosal; GI-IM, gastric and intestinal mixed phenotype intestinal metaplasia; I-IM, solely intestinal phenotype intestinal metaplasia.

with progression of each histological type of classification.[23] Our present data showed 11 (16.7%) and 25 (37.9%) cases to be G and I types, respectively, in early differentiated GC, while 33 (51.6%) and five (7.8%) undifferentiated GC were judged to be of G type and I types. Recently, we reported that 10 (17.9%) and 18 (32.1%) cases were judged to be G type and I type, respectively, in 56 advanced differentiated GC, while this was the case for 22 (18.2%) and 35 (28.9%) of 121 advanced undifferentiated GC using the same epithelial cell markers.[10] Yoshikawa *et al.* suggested that GC cells of differentiated type may be more prone to intestinalization than those of undifferentiated type.[5] Taking into account these analyses of phenotypic expression, we consider that the shift from gastric to intestinal phenotypic expression in the differentiated cancers might occur at an earlier stage than in undifferentiated lesions.

Our present data show that Cdx2 nuclear staining is strongly associated with intestinal phenotypic expression in early GC, compatible with earlier findings.[10,11,33,46] However, Cdx2 nuclear staining was detected in cancerous areas not only where intestinal phenotypic expression was apparent but also in tissue exhibiting only gastric markers or in null phenotypic region, independent of the histological type or depth of invasion. This apparent discrepancy might reflect the intestinal phenotypic epithelial cell markers used in this study. Eda *et al.* have previously suggested that Cdx2 triggers the initiation and development of IM in chronic gastritis from analyses of mRNA levels.[34] We also need to consider the possibility that expression of Cdx2 might precede intestinal phenotypic expression during the shift from gastric to intestinal phenotypic expression with progression of GC.

Bai *et al.*[31] and Seno *et al.*[32] have shown that Cdx2 is associated with differentiated GC with a classification based solely on morphology. In our present study, the rates for Cdx2-positive cases in the early differentiated and undifferentiated types were 60 (90.9%) and 42 (65.6%), respectively, the difference being significant ($P = 0.0005$). We also have shown that intestinal phenotypic expression is observed more frequently in the early differentiated cancers compared with the undifferentiated types, and that Cdx2 is strongly associated with the intestinal phenotypic expression using several phenotypic markers (Tables 1,2). Therefore, the high rate of early differentiated GC with both Cdx2 and the intestinal phenotypic expression supports the consideration that the shift from gastric to intestinal phenotypic expression might occur earlier in such lesions than in the undifferentiated cancers.

It has been suggested that differentiated gastric carcinomas arise from mucosa with IM, but that undifferentiated GC originate from mucosa without IM in view of morphological similarities between each cancer and surrounding mucosa.[1,2,12,13] However, previous studies on phenotypic expression of individual intestinal metaplastic or stomach cancer cells have pointed to several contradictions to this hypothesis.[4,6,9,14–18,20,23,47] In particular, Kawachi *et al.* have shown that the phenotypes of microcarcinomas (defined as lesions <3.0 mm across) and their surrounding mucosa are unrelated,[9] as confirmed for Cdx2 expression and the phenotypes in the present study. We previously presented evidence that each gland is derived from one stem cell in C3H BALB/c chimeras mice, and that individual cancers are derived from single cells with multipotential activity.[48] These GC were confirmed mucin-histochemically to have a gastric phenotypic expression in the chimeras mice.

In conclusion, our data suggest that Cdx2 is expressed at the early stage of gastric carcinogenesis in intestinal phenotypic elements, and could be associated with suppression of expansion of malignant cells. Furthermore, the shift from gastric to intestinal phenotypic expression in the differentiated cancers might occur in the earlier stage than that in the undifferentiated ones. Further study is needed to determine precancerous lesions for GC.

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Eradication of *Helicobacter pylori* induces apoptosis and inhibits proliferation of heterotopic proliferative glands in infected Mongolian gerbils

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Mongolian gerbils infected with *Helicobacter pylori* (*H. pylori*) develop heterotopic proliferative glands (HPGs) in the glandular stomach submucosa. To investigate the effects of *H. pylori* eradication on cell turnover in HPGs, three antibiotics, lansoprazole, amoxicillin and clarithromycin, were administered at 50 or 75 weeks after inoculation of *H. pylori*, and the stomachs were excised for histological examination at 1, 2, 4, 8 or 25 weeks thereafter. The HPGs were classified into gastric type (G-type) and others (GI+I-type), which included both pure intestinal (I-type) and gastric-and-intestinal mixed type (GI-type). Apoptosis and cell proliferation were evaluated by means of TUNEL assay and BrdU labeling, respectively. At 8 weeks post-eradication, apoptotic indices were significantly increased in the eradication group (G-type: 2.5%; GI+I-type: 7.2%) compared to the non-eradication group (G-type: 0.6%; GI+I-type: 2.1%: $P<0.01$), while BrdU labeling indices were significantly decreased (G-type: 1.9%; GI+I-type: 6.8% as compared with 4.3% and 13.2%, respectively, $P<0.01$ for both). At 25 weeks, the apoptotic indices were similarly higher [G-type: 0.4 (eradication group) vs. 0.2% (non-eradication group); GI+I-type: 5.8 vs. 1.1%, both $P<0.01$], and the BrdU labeling indices (G-type: 0.8 vs. 2.2%, $P<0.01$; GI+I-type: 5.1 vs. 11%, $P<0.05$) continued to be lower in HPGs. Furthermore, there were highly significant reductions in the areas of HPGs at 8 and 25 weeks post-eradication. These findings demonstrated that eradication results in apoptosis and reduction of proliferation of HPGs in *H. pylori*-infected gerbils, these lesions thus being apparently reversible through regulation of cell kinetics. (Cancer Sci 2004; 95: 872–877)

*H*elicobacter pylori (H. pylori) infection has been implicated as a major cause of chronic gastritis and a strong risk factor for gastric adenocarcinoma. Epidemiological and experimental studies have demonstrated associations between H. pylori infection and the development of chronic superficial gastritis, atrophic gastritis and intestinal metaplasia.[1–4] Furthermore, we previously reported that Mongolian gerbils infected long-term with H. pylori frequently exhibit heterotopic proliferative glands (HPGs) in the glandular stomach submucosa, and these may disappear on successful eradication of the bacterium.[5] However, the mechanisms underlying such reduction in HPGs remains unclear.

H. pylori alters gastric epithelial cell cycle events in the human, mouse and Mongolian gerbil, with both cell proliferation and cell loss playing important roles.[6–8] Several studies have also provided evidence that cure of H. pylori infection is beneficial in preventing progression of atrophy and intestinal metaplasia.[9,10] Correa et al. showed that a subset of preneoplastic conditions can regress with anti-H. pylori treatment and antioxidant micronutrients supplementation.[11] Recently, Wong et al. presented results from the first randomized clinical trial of H. pylori eradication with cancer as an outcome. Although the study found that H. pylori eradication did not prevent cancer during 7.5 years of follow-up, eradication of H. pylori significantly decreased the development of gastric cancer in a subgroup of H. pylori carriers without precancerous lesions, including gastric atrophy, intestinal metaplasia or dysplasia.[12] To gain insight into the effects of H. pylori eradication on cell turnover in HPGs, we here investigated the levels of apoptosis and proliferation of these lesions in H. pylori-infected gerbils after eradication.

Materials and Methods

Animals and *H. pylori* challenge and eradication. Specific pathogen-free male Mongolian gerbils (Meriones unguiculatus; MGS/Sea) were purchased from Seac Yoshitomi, Ltd. (Fukuoka, Japan) at the age of 7 weeks, and housed in steel cages on hardwood chip bedding in an air-conditioned biohazard room with a 12-h light/12-h dark cycle. Gerbils were given food (Oriental CRF-1; Oriental Yeast Co., Tokyo) and water ad libitum. All experiments and procedures carried out on the animals were approved by the Animal Care Committee of Aichi Cancer Center Research Institute. H. pylori (ATCC 43504, American Type Culture Collection, Rockville, MD) for challenge were grown from freezer stocks for 72 h and harvested in Brucella broth. Samples containing about 1.0×10^8 colony-forming units (0.8 ml) per milliliter were used as the inoculum, as described previously.[13]

For eradication of H. pylori, a "triple therapy" was employed. The drugs, lansoprazole, amoxicillin and clarithromycin, were suspended in 0.5% w/w carboxymethyl cellulose sodium salt solution and administered intragastrically (i.g.) twice a day for 2 days at doses of 10, 3 and 30 mg/kg body weight, respectively.[14]

Experimental protocol. The animals were allocated to experiments I or II (Fig. 1). In experiment I, 40 gerbils were divided into three groups. H. pylori was inoculated into groups 1 (n=21) and 2 (n=9). Group 3 (n=10) received Brucella broth without H. pylori. At week 50, eradication was performed for group 1 animals, which were then sacrificed at 1 (n=5), 2 (n=6), 4 (n=5) and 8 (n=5) weeks thereafter. Animals in groups 2 and 3 were sacrificed after 50 and 58 weeks as controls. In experiment II, 35 gerbils were divided into three groups (4, 5 and 6). At 75 weeks after inoculation of H. pylori, animals in group 4 underwent treatment for eradication, and then were sacrificed after 4 (n=5), 8 (n=5) or 25 (n=5) weeks. Gerbils in groups 5 and 6 were sacrificed after 75 and 100 weeks as controls. For both groups, 5′-bromo-2′deoxyuredine (BrdU) at a dose of 100 mg/kg, was injected intraperitoneally, 60 min before the sacrifice. All animals were subjected to deep

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ether anesthesia after 24 h fasting, laparotomized, and exsanguinated from the inferior vena cava, after which their stomachs were excised.

Histopathological analyses. 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 (HE) or with Alcian blue (pH 2.5)-periodic acid Schiff (AB-PAS) for detection of mucin-containing cells. The HPGs were evaluated and divided into two types, according to the presence or absence of intestinal components. The former were termed the gastric (G) type and the latter included pure I- and mixed GI-types, according to our previously described criteria.[5,15]

Apoptotic cells in the HPGs were detected with an in situ cell death detection kit (Roche Diagnostics, Mannheim, Germany), based on the terminal-deoxynucleotidyl transferase-mediated dUTP nick-end labeling method (TUNEL). After paraformaldehyde-fixed and paraffin-embedded tissue sections had been deparaffinized, specimens were digested with proteinase K at

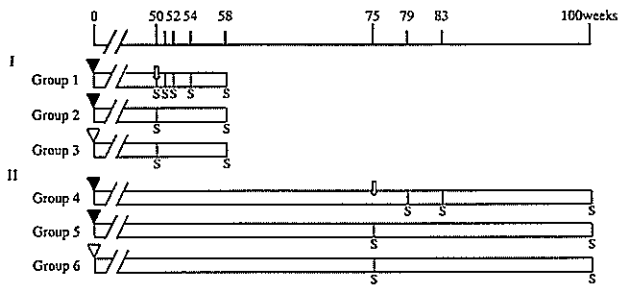

Fig. 1. Experimental design. Animals: 7-week-old male Mongolian gerbils. ▼ *H. pylori* (i.g.), ▽ *Brucella* broth (i.g.), ⇩ eradication. S: sacrifice.

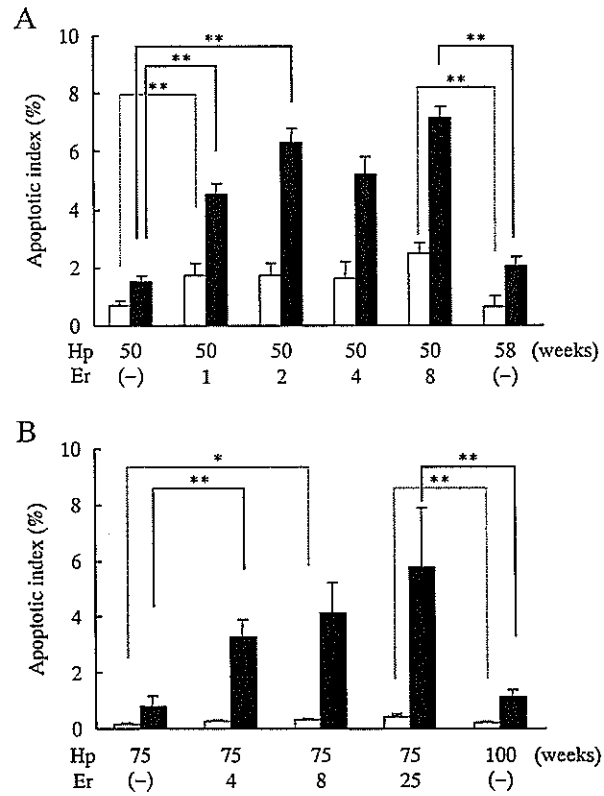

Fig. 3. Effects of eradication on indices of apoptotic cells of HPGs (%, mean±SE). At 8 weeks (A) and 25 weeks (B) post-eradication, the number of apoptotic cells is significantly greater in *H. pylori*-eradicated gerbils than in gerbils without eradication. □ G-type, ■ GI+I-type. * $P<0.05$ and ** $P<0.01$.

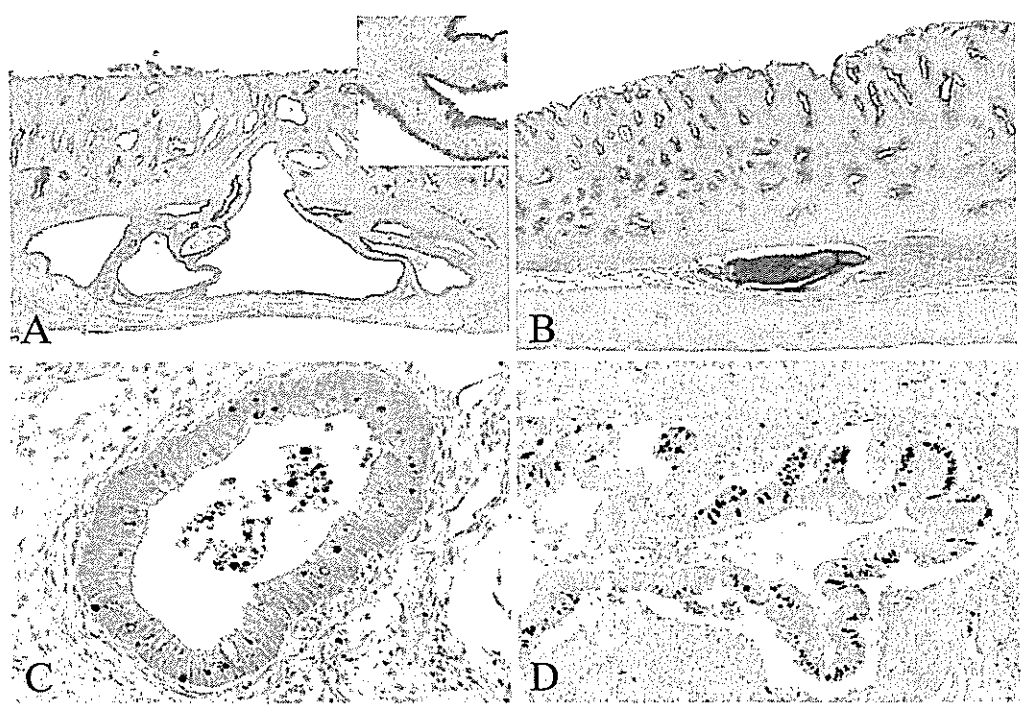

Fig. 2. Histology of heterotopic proliferating glands in *H. pylori*-infected gerbils. A. Gastric-type HPGs with cystic dilation after 50 weeks of *H. pylori* infection (AB-PAS, original magnification ×40). Inset, Higher magnification of HPG lined with PAS-positive foveolar type epithelium (×400). B. HPGs with remnant subserosal mucin at 8 weeks post-eradication (AB-PAS, ×50). C. Note the presence of apoptotic nuclei in intestinal-type HPGs after eradication (TUNEL, ×250). D. Proliferation as demonstrated by BrdU immunostaining in a *H. pylori*-infected gerbil (BrdU, ×125).

37°C for 15 min and washed with phosphate-buffered saline (PBS). Endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide in methanol for 30 min, then the sections were immersed in the terminal deoxynucleotidyltransferase reaction mixture containing enzyme and fluorescein-labeled dUTP at 37°C for 1 h. Anti-fluorescein antibodies conjugated to horseradish peroxidase were applied to the sections to detect labeled nucleotides. Binding was localized with diaminobenzidine and the sections were lightly counterstained with hematoxylin.[16] The BrdU labeling was visualized using a mouse monoclonal anti-BrdU antibody (1:50, DAKO, Kyoto) as described previously.[17]

Four to six sections per animal were prepared for staining of apoptotic cells and BrdU-labeled cells. For randomization, two experienced pathologists (T.T. and M.T.), blinded as to H. pylori treatment, conducted the selection and histopathologic evaluation of sections. The numbers of TUNEL-positive cells and BrdU-labeled cells in the all HPGs were counted (by X.C. and T.T.) by microscopic examination with a 40× objective lens, and indices were determined as the mean percentages of positive cells among total cells of HPGs, where total cells of HPGs were more than 1000. Areas of HPGs were assessed using NIH Image version J1.272 (National Institutes of Health, USA) and values per unit length of slices of glandular stomach (mm^2/cm) were calculated.

Serology. The titers of anti-H. pylori IgG antibodies in serum samples were measured with an enzyme-linked immunosorbent assay, and serum gastrin levels were measured with a radioimmunoassay. The antibody titers were expressed on an arbitrary index, and values greater than the cut-off of 1.5 were considered to be positive for H. pylori infection.[17]

Statistical analyses. Results for apoptosis and BrdU labeling indices, as well as antibody and gastrin levels, were expressed as means±SE. The Bonferroni multiple-comparison test was performed to establish the significance of differences.

Results

The survival rates of all groups were >95%. The stomach epithelium contained HPGs in all gerbils at 50 and 75 weeks after H. pylori infection (groups 1, 2, 4 and 5) (Fig. 2A). HPGs developed only in the antrum and junctional region of the antrum and the body showing pseudopyloric metaplasia. No HPGs were observed in fundic mucosa in this experiment. In this study, no animals had developed adenocarcinomas by the end of the experiments.

Normal epithelial cell apoptosis and proliferation in uninfected gerbils. Normal epithelial cell apoptosis and proliferation were assessed in group 3. Apoptotic indices for the corpus, antrum, jejunum and colon were 0.5±0.1%, 0.8±0.2%, 1.3±0.4% and 0.7±0.3% (mean±SE), respectively, at 50 weeks. BrdU labeling indices were 2.6±0.5%, 5.2±1.0%, 9.6±1.9% and 5.9±1.5%, respectively. There were significant differences between gastric and jejunum epithelial cells.

Effect of *H. pylori* eradication on apoptosis in epithelial cells in HPGs. Apoptotic indices for G-type HPGs were 1.7±0.4% (51 weeks), 1.7±0.5% (52 weeks), 1.6±0.6% (54 weeks) and 2.5±0.4% (58 weeks) in group 1, 0.7±0.2% (50 weeks), 0.6±0.4% (58 weeks) in group 2, 0.3±0.04% (79 weeks),

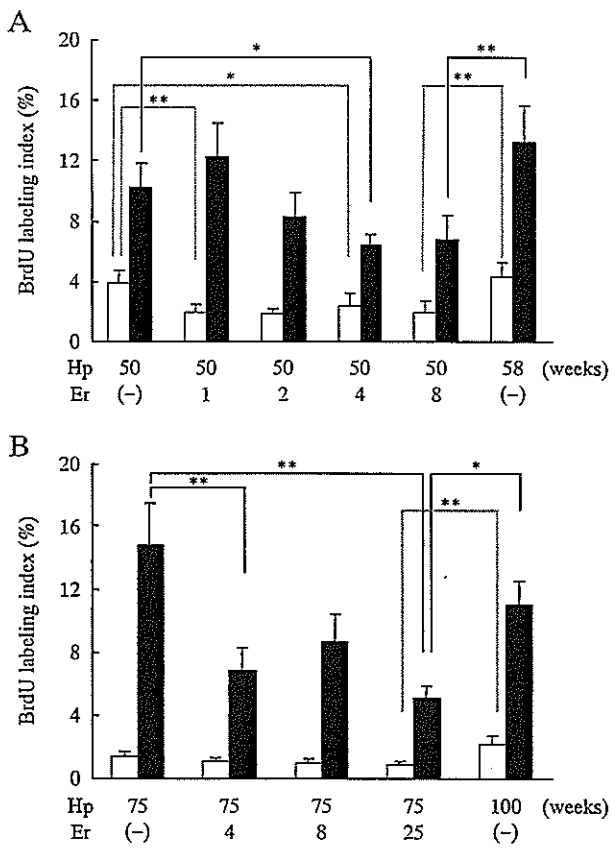

Fig. 4. Effects of eradication on the number of BrdU-labelled cells of HPGs (%, mean±SE). At 8 weeks (A) and 25 weeks (B) post-eradication, the numbers of labelled cells are significantly lower in *H. pylori*-eradicated gerbils as compared with gerbils without eradication. □ G-type, ■ GI+I-type. * $P<0.05$ and ** $P<0.01$.

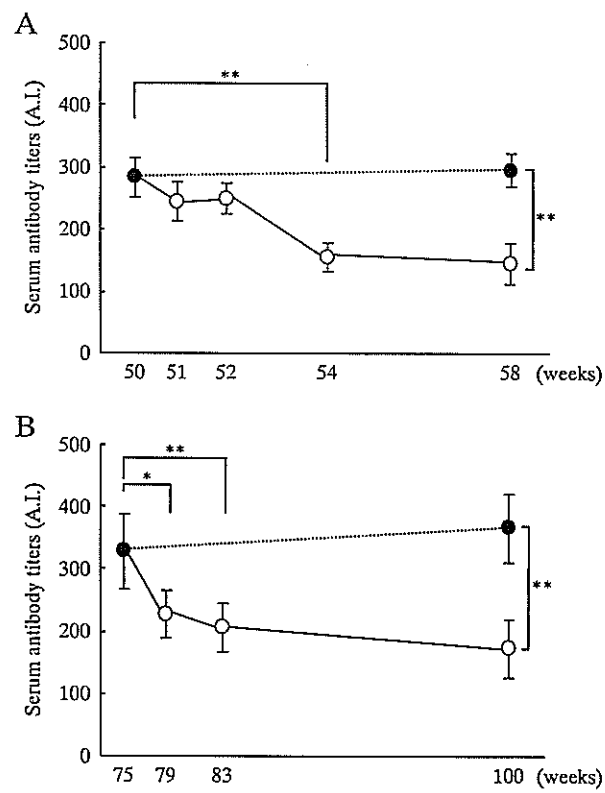

Fig. 5. Changes in serum anti-*H. pylori* IgG titers of gerbils after eradication. Experiments I (A) and II (B) (mean±SE). At 8 and 25 weeks post-*H. pylori* eradication, serum anti-*H. pylori* IgG titers were significantly decreased in the *H. pylori*-eradicated gerbils as compared to the non-eradication groups. ● non-eradication, ○ eradication with lansoprazole, amoxicillin and clarithromycin. A.I.: arbitrary index. * $P<0.05$ and ** $P<0.01$.

0.3±0.08% (83 weeks), 0.4±0.1% (100 weeks) in group 4, and 0.2±0.03% (75 weeks), 0.2±0.05% (100 weeks) in group 5. Indices for GI+I-type HPGs were 4.5±0.7% (51 weeks), 6.3±1.4% (52 weeks), 5.2±1.3% (54 weeks), 7.2±1.2% (58 weeks) in group 1, 1.5±0.5% (50 weeks), 2.2±0.5% (58 weeks) in group 2, 3.3±0.6% (79 weeks), 4.1±1.1% (83 weeks), 5.8±2.1% (100 weeks) in group 4, and 0.8±0.2% (75 weeks), 1.1±0.3% (100 weeks) in group 5. Apoptotic indices of HPGs in the eradication groups, as shown in Fig. 2C, were significantly higher than in the non-eradication groups ($P<0.01$) (Fig. 3, A and B).

BrdU labeling index. BrdU labeling indices of G-type HPGs were 1.9±0.6% (51 weeks), 1.8±0.4% (52 weeks), 2.4±0.8% (54 weeks), 2.0±0.7% (58 weeks) in group 1, 3.9±0.9% (50 weeks), 4.3±1.0% (58 weeks) in group 2, 1.0±0.5% (79 weeks), 1.1±0.7% (83 weeks), 0.8±0.5% (100 weeks) in group 4, and 1.4±0.4% (75 weeks), 2.2±0.5% (100 weeks) in group 5. Indices of GI+I-type HPGs were 12.3±2.2% (51 weeks), 8.3±1.6% (52 weeks), 6.4±0.7% (54 weeks), 6.8±1.6% (58 weeks) in group 1, 10.2±1.6% (50 weeks), 13.2±2.4% (58 weeks) in group 2, 6.8±1.2% (79 weeks), 8.8±2.0% (83 weeks), 5.1±0.8% (100 weeks) in group 4, and 14.8±5.3% (75 weeks), 11.0±1.6% (100 weeks) in group 5. BrdU labeling indices in eradication groups were significantly decreased as compared to non-eradication groups ($P<0.01$) (Figs. 2D, 4, A and B).

Change of anti-*H. pylori* IgG titer and gastrin level. The anti-H. pylori IgG titer and gastrin level were significantly higher in non-eradication groups compared to those in the eradication groups (Figs. 5 and 6).

Change of areas of HPGs. As shown in Fig.7, there were highly significant reductions in the areas of HPGs between the eradicated and non-eradication groups (Fig. 7, A and B).

Discussion

HPGs usually arise in response to long-term H. pylori infection in gerbils, exhibiting hyperplastic changes and variable degrees of multifocal cystic dilatation, and their characteristics have already been described in detail.[5] Our present data provide clear evidence that the apoptotic indices of HPGs significantly increase after eradication of the bacteria, while the BrdU labeling indices are significantly decreased. While continual expansion of HPGs was observed in the glandular stomachs of H. pylori-infected gerbils without eradication, a significant reduction in size was evident at 4–8 weeks post-eradication, subsequent to the alteration in the levels of cell apoptosis and proliferation. A decrease of serum anti-H. pylori IgG titer accompanied eradication, as expected, and acid secretion may have resumed, contributing to the recovery of gastrin level.[18] Our data suggest that changes of cell turnover precede the reduction of HPGs area and therefore are causal for reversibility, upon elimination of the bacteria.

Previous studies have provided evidence that H. pylori infection induces apoptosis and cell proliferation in gastric epithelium, and these return to normal after eradication of the infection in humans.[19,20] Similar results have also observed in glandular epithelial cells in Mongolian gerbil models.[21,22] The alterations of epithelial cell turnover are probably associated with activation of certain caspases, the mitochondrial apoptotic pathway and vacuolating cytotoxin (VacA).[23,24] Recent studies indicated that the cagPAI-positive H. pylori strain ATCC43504

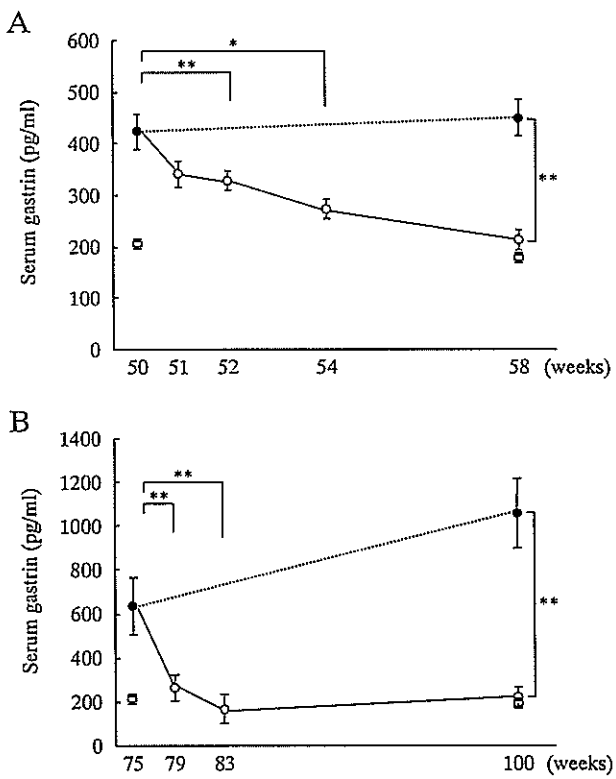

Fig. 6. Changes in serum gastrin level in experiments I (A) and II (B) (mean±SE). Note the significantly falls in the *H. pylori*-eradicated gerbils as compared to the non-eradication groups. □ uninfected control, ● non-eradicated, ○ eradication with lansoprazole, amoxicillin and clarithromycin. * $P<0.05$ and ** $P<0.01$.

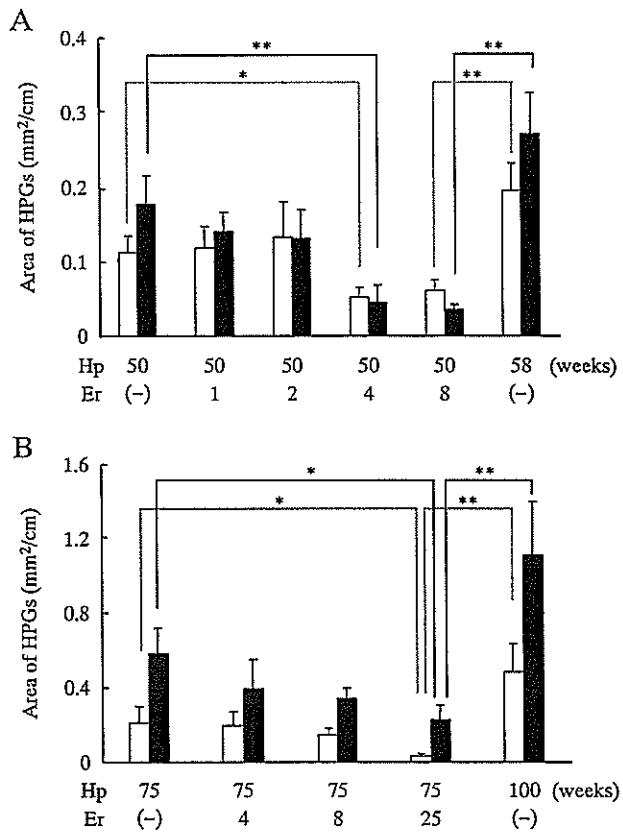

Fig. 7. Areas of HPGs (mean±SE) at 8 weeks (A) and 25 weeks (B) post-eradication. □ G-type, ■ GI+I-type. * $P<0.05$ and ** $P<0.01$.

activates Smad 5 mRNA expression, which is essential for H. pylori-induced apoptosis in gastric epithelial cells.[25] On the other hand, anti-apoptotic effects of H. pylori infection mediated by NFκB activation and decreased expression of p27 (kip1) have also been observed in in vitro studies.[26,27] In this study, we focused on HPG, heterotopic hyperplastic changes, which are frequently induced by long-term H. pylori infection in gerbils. The present data showed the apoptosis indices of HPGs to be significantly increased by eradication of H. pylori, compared with non-eradicated animals. Therefore, we consider that the anti-apoptotic effects of H. pylori infection were dominant in the development of HPGs. The alterations of cell turnover may quite different between pathologic HPGs and normal epithelial homeostasis. Further studies on the molecular pathogenesis may be helpful to clarify the differences in cell kinetics between gastric epithelial cells and cells in HPGs.

Interestingly, our data show that the apoptotic and BrdU labeling indices of GI+I-type HPGs were significantly higher than those of G-type HPGs (2- to 5-fold difference) in both the H. pylori eradication and non-eradication groups. This is similar to the difference between normal gastric and intestinal epithelial cells in Mongolian gerbils. Furthermore, GI+I-type HPGs appear likely to be more sensitive to eradication than the G-type HPGs, given the remarkable differences in proliferation ability and biological characteristics between the two phenotypes (Fig. 3, A and B; Fig. 4, A and B). HPGs, which normally show gradual intestinalization with a shift from gastric to intestinal phenotypic expression, were earlier found to be obviously reduced after eradication.[5] Intestinal metaplasia of the stomach is usually considered as a risk factor for development of intestinal-type tumors, but the mechanisms involved are still unclear. In the present study, intestinalization of the HPGs was considered as an adaptive response to H. pylori infection, and eradication not only reduced the areas of HPGs, but also contributed to a reversed phenotypic shift from GI+I- to G-type expression.[5,28]

With regard to the problem of how H. pylori eradication reverses HPGs, two questions arise. First, are HPGs themselves totally reversible if the causative stimulus is removed? Second, does intestinalization of HPGs represent a non-reversible path, due to somatic mutation in stem cells or is it only the consequence of epigenetic events?[29,30] Eradication of H. pylori may not reverse all HPGs, but may accelerate their decline and suppress further development by removing the stimulus.

In conclusion, the present results suggest that eradication of H. pylori increases apoptosis and reduces the proliferative ability of HPGs, especially of the GI+I-type, in the glandular stomach submucosa of gerbils. This then results in reduced areas of HPGs after eradication, underlining the idea that removal of H. pylori may be a useful preventive approach. The apparent increase in apoptosis observed after H. pylori eradication is presumably the result of a complex interplay between bacterial and host factors. However, molecular markers have not been thoroughly explored with regard to the pathogenesis of the lesions in gerbils, and longer follow-up is now necessary to confirm the long-term benefit of eradication treatment.[31]

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Sox2 expression in human stomach adenocarcinomas with gastric and gastric-and-intestinal-mixed phenotypes

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Sox2 expression in human stomach adenocarcinomas with gastric and gastric-and-intestinal-mixed phenotypes

Aims: Other than ectopic expression of intestinal transcription factors, *Cdx1* and *Cdx2*, the molecular mechanisms underlying gastric and intestinal phenotypes of human stomach adenocarcinomas have yet to be clarified in detail. We have reported that Sox2, an HMG-box gastric transcription factor, is expressed in normal gastric mucosa and down-regulated in intestinal metaplasia.

Methods and results: We analysed mRNA levels of *Sox2* and other differentiation markers in 50 surgically resected stomach adenocarcinomas, immunohistochemically classified into gastric (G), gastric-and-intestinal (GI)-mixed, solely intestinal (I), and null (N) types. Sox2 was found to be transcribed in G and GI-mixed type adenocarcinomas in accordance with MUC5AC and MUC6 expression, while Cdx1 and Cdx2 were up-regulated in GI-mixed and I types along with the expression of MUC2 and villin. In the N type, both gastric and intestinal transcription factors were suppressed. Immunohistochemistry confirmed expression of Sox2 in MUC5AC+ lesions and Cdx2 localization together with MUC2. A stomach adenocarcinoma cell line, KATOIII, demonstrated both MUC5AC and Sox2, although MUC5AC mRNA was not detected in the Sox2+ AGS cell line.

Conclusions: Sox2 may play an important role in maintaining a gastric phenotype in stomach cancers as well as in normal tissue, in cooperation with other cofactor(s).

Keywords: Cdx1, Cdx2, gastric-and-intestinal-mixed type, gastric transcription factor, HMG box, homeobox genes, intestinal transcription factor, Sox2

Abbreviations: G, gastric; GI, gastric and intestinal; HMG, high-mobility group; IM, intestinal metaplasia; N, null; RT-PCR, reverse transcriptase-polymerase chain reaction

Introduction

Human gastric carcinomas have been classified by Lauren into two major groups, the 'intestinal' and 'diffuse' types,[1] which, respectively, nearly correspond to the 'differentiated' and 'undifferentiated' types of Nakamura *et al.*[2] and Sugano *et al.*[3] However, the above-mentioned classifications are inadequate for studies of histogenesis of gastric carcinomas and phenotype expression at the cellular level, because they confuse an intestinal phenotype with a 'diffuse' structure and a gastric phenotype with the 'intestinal' type of Lauren.[4] The phenotypic expression of stomach cancer cells of each histological type can be clearly classified into gastric and intestinal epithelial cell types by immunohistochemistry using gastric and

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intestinal epithelial cell markers such as MUC5AC, MUC6, MUC2 and villin.[4] It has been suggested that 'intestinal' type carcinomas arise in intestinalized mucosa, whereas the 'diffuse' type develops from the gastric mucosa proper.[5] This hypothesis is based on morphological similarities between cancers and intestinal metaplasia (IM), and on the results of comparisons of carcinomas and surrounding mucosa.[6] However, previous studies of phenotypic expression of individual intestinal metaplastic or stomach cancer cells have revealed several contradictions to this hypothesis.[7–9] It is widely thought that the phenotypic expression of tumour cells resembles that of the tissue of origin. We have previously shown that stomach cancers at an early stage, independent of the histological type, mainly consist of gastric phenotypic cancer cells and a shift from gastric to intestinal phenotypic expression is then observed with progression in experimental animal models.[10–13] It is clearly of interest to determine what changes in gene expression are associated with this phenotypic shift.

Candidate genes controlling gastric and intestinal phenotypes include several transcription factors.[14] The *caudal*-related homeobox genes (Cdx) are important for the maintenance of intestinal epithelial cells[15–18] and there have been several reports of Cdx1 and Cdx2 expression in human stomach carcinomas[19,20] and in IM.[18,21–26] In contrast to intestinal regulatory factors, little is known about factors controlling the gastric phenotype. One candidate is encoded by the *cSox2* gene, a member of the transcription factor family containing an *Sry*-like high-mobility group (HMG) box, which demonstrates localized expression in the chicken stomach.[27] Since expression of Sox2 has been shown to be confined to the fundic and pyloric regions of the stomach and undetectable from the duodenum though to the rectum, it could be a key molecule for gastric differentiation in the gastrointestinal tract of mammals.[26,28] We have recently reported Sox2 transcripts to be down-regulated in a stepwise manner from the GI-mixed type toward complete I type lesions in the human stomach, in association with decrease of gastric mucin, MUC5AC.[26] Thus, we hypothesize that Sox2 may regulate the gastric phenotype in adenocarcinoma cells in association with mucin expression.

Li *et al.*[29] have already demonstrated Sox2 expression in G-type stomach adenocarcinomas using an immunohistochemical approach. In the present study, we document expression of *Sox2* transcripts in G- and GI-mixed-type adenocarcinomas, as assessed by relative quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) methods. We also utilized stomach adenocarcinoma cell lines and suggest the existence of other cofactor(s) cooperating with Sox2 protein.

Materials and methods

HUMAN TISSUES

This study using human tissue was conducted with approval from the Ethical Review Board of Aichi Cancer Centre. Fifty specimens of human stomach adenocarcinomas were surgically resected and examined after obtaining informed consent. Advanced carcinomas were selected to avoid taking surrounding normal mucosa and this was confirmed by histology.

HISTOLOGY AND IMMUNOHISTOCHEMISTRY

Histological and immunohistochemical procedures were performed as described.[26] Briefly, tissue samples were fixed in 10% buffered formalin, dehydrated, and embedded in paraffin. Sections were stained with haematoxylin (Merck, Darmstadt, Germany) and eosin (Muto Pure Chemicals, Co., Ltd, Tokyo, Japan) (H–E) for histopathological examination. For the immunohistochemical detection, the antibodies listed in Table 1 were applied, and binding was visualized with 3,3′-diaminobenzidine (DAB) (Dojindo Laboratories, Kumamoto, Japan). Sections were then counterstained with haematoxylin (for MUC5AC, MUC6, MUC2, villin, and Cdx2) or light green SF yellowish (Chroma-Geselschaft Schmid GmbH & Co., Kongen, Germany) (for Sox2). The results of each antibody staining were evaluated in terms of the percentage of stained cancer cells, with 10% and above considered positive, as previously described.[19,20] Micrographs were taken using an Axioplan microscope equipped with AxioCam HRc (Zeiss, Germany), with or without differential interference contrast.

CLASSIFICATION OF CANCERS

MUC5AC and MUC6 are markers of the gastric epithelial cell phenotype, whereas MUC2 and villin are typical of intestinal epithelial cells.[4,19,20,26] Two independent pathologists (T.T. and M.T.) judged the histology and immunohistochemical reactivity of the phenotypic markers. Gastric cancers, in which more than 10% of the section area consisted of at least one gastric or intestinal epithelial cell phenotype, were classified as gastric (G type) or intestinal (I type) phenotype cancers, respectively. Those which showed both gastric and intestinal phenotypes were classified as gastric-and-intestinal-mixed phenotype (GI-mixed

Table 1. Antibodies and cell types

Antigen	Clone	Target cells	Source	Dilution
MUC5AC	CLH2	Gastric foveolar epithelial cells	Novocastra Laboratories, Newcastle upon Tyne, UK	1 : 500
MUC6	CLH5	Pyloric gland cells and mucous neck cells	Novocastra Laboratories	1 : 500
MUC2	Ccp58	Goblet cells in intestine	Novocastra Laboratories	1 : 500
Villin	12	Intestinal absorptive cells	Transduction Laboratories, Lexington, KY, USA	1 : 20 000
Sox2	Polyclonal	Gastric epithelial cells	Chemicon, Temecula, CA, USA	1 : 200
Cdx2	CDX2-88	Intestinal epithelial cells	BioGenex, San Ramon, CA, USA	1 : 50

type) cancers, while those showing neither gastric nor intestinal phenotype expression were grouped as null type (N type).

TOTAL RNA ISOLATION AND QUANTITATIVE RT-PCR

Total RNA was isolated with TRIzol reagent (Roche Diagnostics, Mannheim, Germany) and first-strand cDNA synthesis was performed using the Thermoscript RT-PCR System (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Real-time relative quantitative PCR of gastrointestinal markers (*MUC5AC*, *MUC6*, *MUC2*, and *villin*) and gastric and intestinal transcription factors (*Sox2*, *Cdx1*, and *Cdx2*) was performed with the LightCycler system (Roche Diagnostics), using *acidic ribosomal phosphoprotein PO (ARP)/36B4* as an internal control.[26,30] The PCR primers employed are listed in Table 2.

QUANTITATIVE ANALYSIS

Quantification was performed as earlier established using an internal control, with values expressed as percentages of those in G-type adenocarcinomas for *MUC5AC*, *MUC6*, and *Sox2* (set as 100%). Similarly, regarding *MUC2*, *villin-1*, *Cdx1*, and *Cdx2*, expression levels were relative to those in I-type carcinomas. The values for corrected crossing points were used for statistical analyses, applying the Mann–Whitney *U*-test as previously described.[26,30]

CELL CULTURE AND SEMIQUANTITATIVE RT-PCR

Stomach adenocarcinoma cell lines including KATOIII, MKN45, AGS, HSC57 and MKN28 were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Total RNA isolation and semiquantitative PCR were performed basically as described above except for the PCR cycle numbers: 32 cycles for Sox2 and 30 cycles for MUC5AC and ARP. PCR products were visualized with ethidium bromide without SYBR green.

Results

EXPRESSION OF GASTRIC AND INTESTINAL EPITHELIAL CELL MARKERS IN STOMACH CANCERS

Representative results of immunohistochemical analyses for differentiation markers in gastric cancers are shown in Figures 1 and 2A,B,D,E). Taking into account the combinations of expression of MUC5AC, MUC6, MUC2, and villin, the lesions were divided phenotypically into 11 G, 10 GI-mixed, 12 I, and 17 N. Immunostaining of Sox2 and Cdx2 revealed that the localization of these transcription factors correlated well with staining for MUC5AC and MUC2, respectively (Figures 1C,F and 2C,F)

EXPRESSION OF GASTRIC AND INTESTINAL MARKERS mRNA IN STOMACH ADENOCARCINOMAS

Total RNAs were isolated from the gastric cancers characterized above and subjected to quantitative analysis of the gastric and intestinal markers as well as the transcription factors. For the gastric markers, Sox2, MUC5AC, and MUC6, the average values for G-type adenocarcinomas were set at 100% and data for other types expressed as relative values. Relative average expression levels (Ave-SE – Ave + SE) for Sox2 were 100.0% (62.8–159%), 100.6% (62.2–163%), 14.4% (8.09–25.6%) and 13.5% (7.1–25.7%) in G-, GI-mixed-, I- and N-type stomach adenocarcinomas, respectively. The figures for MUC5AC were 100.0 (44.6–225%), 73.0% (21.3–250%), 1.17% (0.33–4.1%) and 1.57% (0.41–6.1%), and for MUC6 were

Table 2. Primer sequences for real-time reverse transcriptase-polymerase chain reaction

Target genes	Orientation	Sequence	Product length (bp)	Gene bank accession nos
Sox2	Upper Lower	CGCCCCCAGCAGACTTCACA CTCCTCTTTTGCACCCCTCCCATTT	170	Z31560
Cdx1	Upper Lower	AGCGCAAAGTGAACAAGAAGAAACAG GGGGCTATGGCAGAAACTCCTCT	177	U51095
Cdx2	Upper Lower	TCAGCCAGGTCCTCTGAGAA GCCTGGAATTGCTCTGCC	170	U51096
MUC5AC	Upper Lower	CAGAAATCCAGGACAACCAC AACAGGGCTCGGAGTAGTTTTA	192	AJ298317
MUC6	Upper Lower	TGCCCACTGTCCAACACC CTCTGGGGATCTCCTCTTCTCT	192	U97698
MUC2	Upper Lower	CCATTCTCAACGACAACCCCTACTACCCC TCCAATGGGAACATCAGGATACATGGTGGC	189	L21998
Villin-1	Upper Lower	AGGGGAATCTGGTGGTGAGGGAAGT GGTGGGTACTGCTTGGCTTTGATGAA	193	XM_010866
ARP	Upper Lower	GCAGACAATGTGGGCTCCAAGCAGA TCCCCCGGATATGAGGCAGCAGTTT	160	M17885

ARP, acidic ribosomal phosphoprotein PO.

100.0% (30.2–331%), 83.7% (27.1–258%), 2.13% (0.84–5.41%) and 2.51% (0.78–8.1%). Sox2 transcripts in I- and N-type adenocarcinomas were significantly down-regulated compared with the G and GI-mixed cases ($P < 0.05$) (Figure 3), in parallel with MUC5AC and MUC6 transcription.

For the intestinal markers Cdx1, Cdx2, MUC2 and villin-1, the average levels in I-type carcinomas were set at 100% and those in other types again expressed as relative values. For Cdx1 they were 1.88% (0.67–5.3%), 5.3% (1.7–16.3%), 100.0% (50.7–197%) and 1.5% (0.70–3.1%) in G, GI-mixed, I and N types, respectively, and for Cdx2, 11.2% (5.7–21.9%), 51.7% (27.5–97.4%), 100.0% (52.9–188.7%) and 23.4% (14.7–37.2%). The respective figures for MUC2 were 6.3% (2.0–20.1%), 76.8% (20.7–285.1%), 100.0% (37.7–266.5%) and 2.5% (1.1–5.5%), and finally for villin-1, 45.0% (31.3–64.7%), 45.3% (23.8–86.3%), 100.0% (69.6–145%) and 15.4% (8.5–27.8%). Cdx1 was predominant only in the I type whereas Cdx2 was up-regulated in both GI-mixed and I types. The expression of MUC2 and villin generally resembled that of Cdx2. Relatively higher expression of villin-1 was noted in G-type carcinoma compared with other intestinal markers. In the N type, all of these markers were significantly suppressed.

EXPRESSION OF MUC5AC AND SOX2 IN STOMACH ADENOCARCINOMA CELL LINES

To analyse further the association of Sox2 and MUC5AC expression, we utilized stomach adenocarcinoma cell lines. KATOIII expressed both Sox2 and MUC5AC, whereas in AGS only transcripts of Sox2 proved detectable. MKN45 showed a weak Sox2 band and no MUC5AC message. HSC57 and MKN28 did not express either of the messenger RNAs (Figure 4).

Discussion

In the present study, analysis of the expression level of a gastric transcription factor, *Sox2*, in stomach adenocarcinomas by a quantitative RT-PCR method demonstrated transcripts in both G-type and GI-mixed-type adenocarcinomas, in close accordance with data for the gastric mucins, MUC5AC and MUC6. Inversely, Sox2 and the two mucins were all down-regulated in parallel in I- and N-type cancers. With Cdx1 and Cdx2, up-regulation was noted in GI-mixed-type and I-type adenocarcinomas, harbouring the intestinal mucin, MUC2, and the villin structural protein typical of the small intestine. In the N type, minimal amounts of differentiation markers were apparent. The results thus

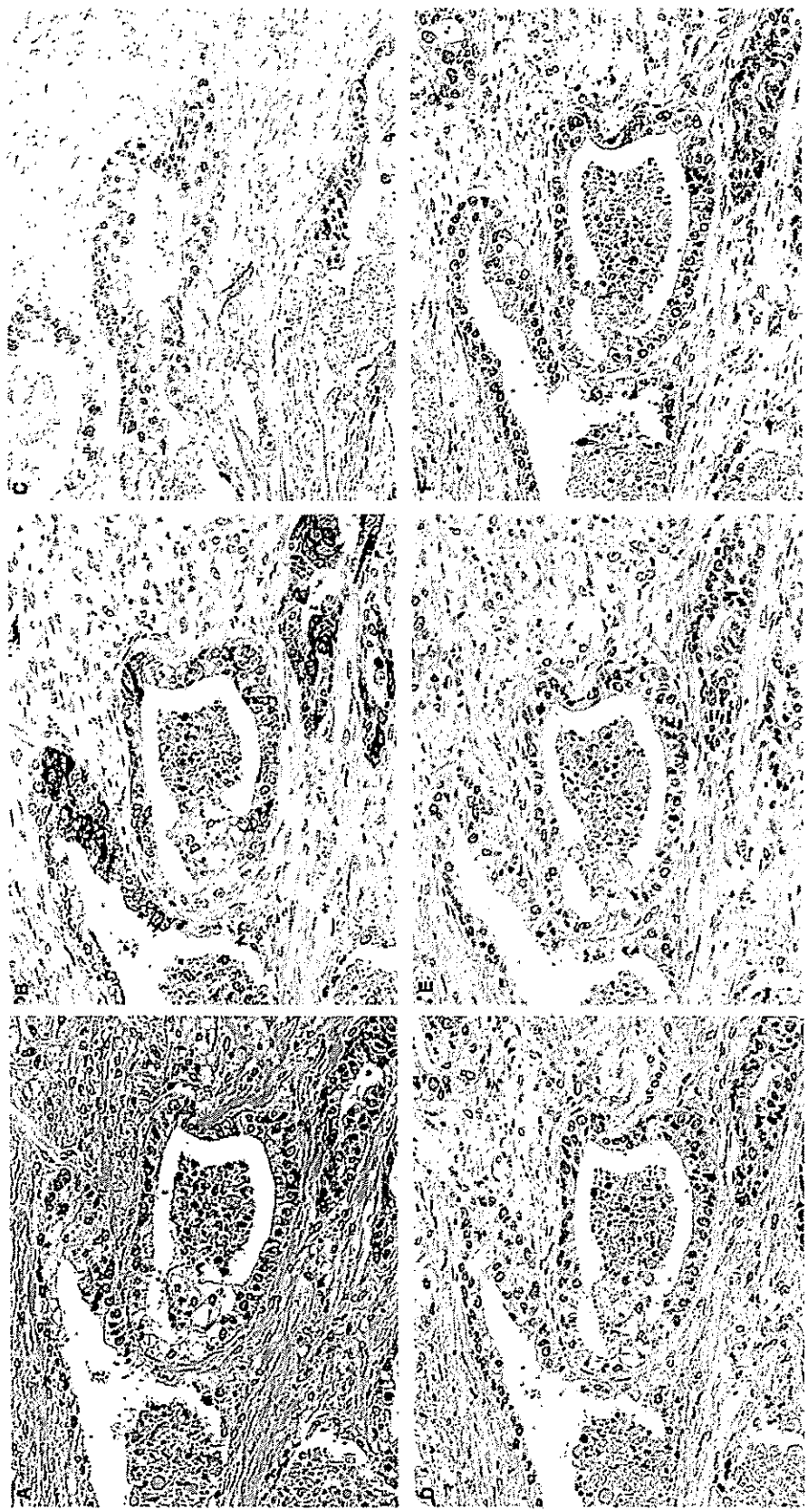
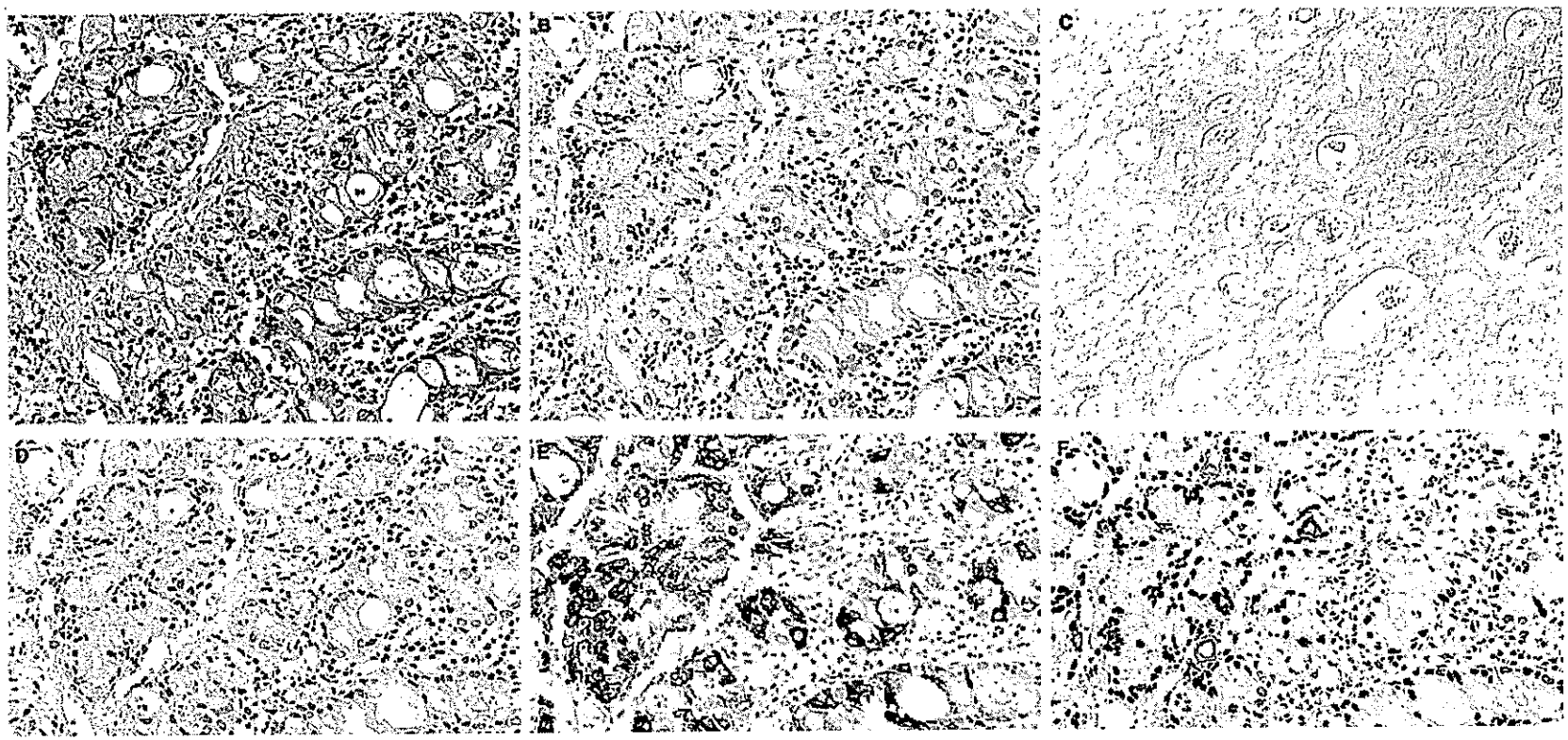


Figure 1. Histology of a gastric (G) type stomach adenocarcinoma. A, H–E. B, MUC5AC. C, Sox2. D, MUC6. E, MUC2. F, Cdx2.


A
B
C
D
E
F

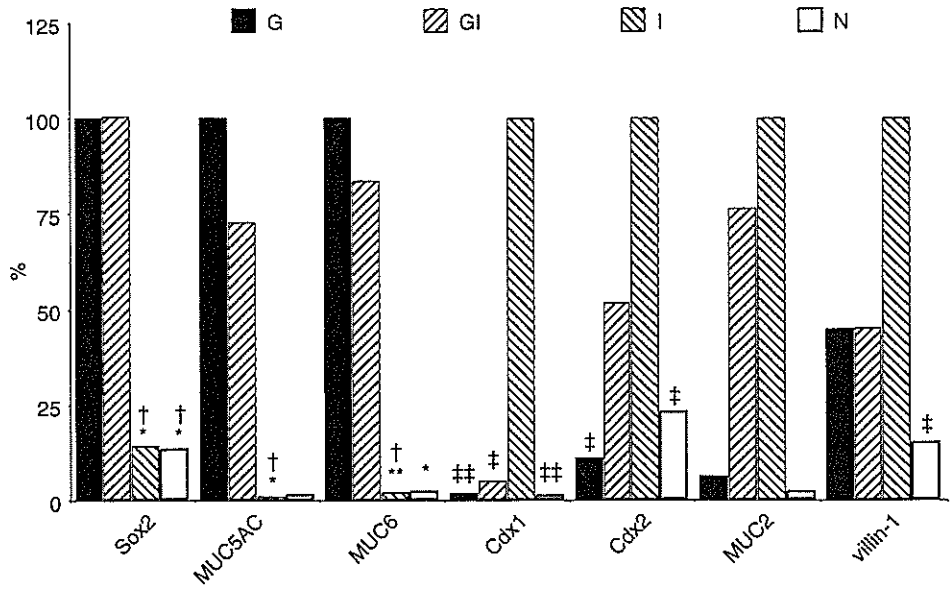


Figure 3. Relative expression levels of gastric and intestinal markers in stomach adenocarcinomas. *$P < 0.05$ and **$P < 0.01$ compared with G-type and †$P < 0.05$ with GI-type carcinomas for Sox2, MUC5AC and MUC6. ‡$P < 0.05$ and ‡‡$P < 0.001$ versus I-type carcinomas for Cdx1, Cdx2, MUC2 and villin-1. The values were set at 100% for Sox2, MUC5AC and MUC6 in the G type and for Cdx1, Cdx2, MUC2 and villin-1 in the I type.

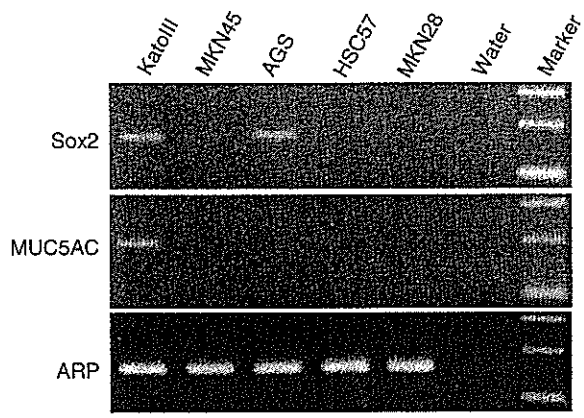


Figure 4. Expression of Sox2 and MUC5AC transcripts in stomach adenocarcinoma cell lines. ARP, acidic ribosomal phosphoprotein PO.

provide further evidence in favour of utilizing such markers in immunohistochemical and molecular biological studies.[31]

Intestinalization is commonly observed in human gastric cancers. Yamachika *et al.*[32] and Bamba *et al.*[33] found signet ring cell carcinomas at an early stage to consist mainly of G-type cancer cells, with a phenotypic shift from G-type to I-type expression accompanying an increase in the depth of invasion. In addition, Yoshikawa *et al.*[34] revealed over 40% of early-stage Lauren's 'intestinal' type carcinomas to consist mainly of G-type cancer cells and again found a phenotypic shift from G to I type with progression. The mechanisms driving transdifferentiation characterized by changes in morphological and mucin histochemical parameters, presumably involve switching of expression of transcription factors such as homeobox genes. Indeed, *Cdx1* and *Cdx2* have been reported to emerge in intestinal metaplastic glands[18,26,35] as well as I-type adenocarcinomas.[19,20] Eda *et al.*[25] reported that expression of *Cdx2* precedes that of *Cdx1* during the progression of IM. In the GI-mixed-type adenocarcinomas in this study, the transcription level of Cdx2 was 51.7% of that in the I type, whereas the respective figure for Cdx1 was only 5.3%. Thus, expression of Cdx2 may precede that of Cdx1 in adenocarcinomas during their intestinalization. Expression of Cdx1 and Sox2 might be mutually exclusive, whereas in the case of Cdx2 independence from Sox2 transcriptional regulation seems more likely. The expected patterns were also observed for *MUC2* and *villin*, although the latter was already expressed at 45% of the level observed in G-type cancers, which might exhibit metaplastic absorptive cell characteristics among gastric mucous cells. This kind of phenomenon has also been reported in IM,[26] which cannot usually be recognized with routine H–E histological analysis due to lack of goblet cells, but can be detected with immuno- and enzyme histochemical staining for intestinal alkaline phosphatase in mouse stomach.[36]

To analyse the shift from a gastric to an intestinal phenotype, one should also focus on gastric transcription factors, like the *Sox* gene family,[37] which consists of 10 subgroups according to HMG box homology. Those of group B1, including Sox1, Sox2, and Sox3, are important for gut development in mice.[38] *In situ*

analysis of the chicken *cSox2* gene has demonstrated localized expression in the embryonic endoderm and transcripts appear before commencement of morphogenesis and cytodifferentiation in the rostral gut epithelium from the pharynx to the stomach. The caudal limit of *cSox2* expression coincides with the region competent for proventricular differentiation and the rostral limit of the domain of *CdxA*.[27] In the human digestive tract, *Sox2* expression is found in stomach epithelium, including fundic and pyloric mucosae, but is very low in intestine, as observed in the chicken.[26] Sakamoto *et al.*[39] observed the localization of *cSox2* transcripts during the development of chicken proventriculus (glandular stomach) utilizing an *in situ* hybridization method and found it to be more strongly expressed in luminal epithelial cells than in glandular epithelial cells, inversely correlating with the expression pattern of embryonic chicken pepsinogen (ECPg). Since cSox2 has been found to down-regulate luciferase activity downstream of the ECPg promoter, it may be a transcription repressor of ECPg. In the human stomach, immunohistochemical analysis revealed Sox2 localization in the foveolar epithelial cells,[26] although expression of the protein in fundic and pyloric gland cells is equivocal, as documented by ourselves[26] and by Li *et al.*[29] Considering the findings for cSox2 and ECPg in the chicken stomach, lower amounts of Sox2 in human fundic and pyloric glands than in foveolar epithelial cells is perhaps not unexpected. In IM, *Sox2* transcripts begin to decrease and gradually disappear with progression from the GI-mixed type to the pure I type and inversely correlating with increases in *Cdx1* and *Cdx2*. Thus, it is possible that Sox2 negatively regulates Cdx1 and Cdx2 expression or *vice versa*. As with the relationships of *MUC2/villin* and *Cdx1/2*, *Sox2* may stimulate the expression of gastric differentiation markers including *MUC5AC*, but repress others such as *MUC6* and *pepsinogen* as suggested by the chicken system.[39]

During lens development in vertebrates, the orchestration of multiple transcriptional regulators is essential for fate determination and terminal differentiation. In early development, Pax6, Sox2 and Six3 are expressed in head ectoderm, while L-maf, Prox1 and crystallin genes are expressed at a later stage in the lens placode in a more restricted fashion.[40] Pax6 initiates lens development by forming a molecular complex with Sox2 on the lens-specific enhancer elements, the delta-crystallin minimal enhancer DC5.[41] Thus, the Sox2 transcription factor in the stomach may require other cofactors to stimulate transcription of gastric mucin and other functional genes. In our present *in vitro* analysis, KATOIII cells appeared to harbour both *Sox2* and *MUC5AC* transcripts. On the other hand, AGS featured only *Sox2* and lacked *MUC5AC* mRNA. This indicates the presence of other cofactor(s) that drive *MUC5AC* transcription. COS-7 cells may possess such putative factors since they have been found to express MUC5AC upon transfection of Sox2 cDNA.[29]

IM has been extensively studied as a putative preneoplastic lesion[42–51] in human stomach, although its significance remains controversial.[4,52,53] GI-mixed-type carcinomas may develop by progression of G types with intestinalization. N types, in contrast, could be directly derived from the G type by simple loss of the gastric phenotype. This hypothesis is consistent with previous reports.[20,23] It should be noted that GI-mixed type and I types, expressing Cdx2, have a better prognosis than do their G and N counterparts.

In conclusion, the present study has demonstrated expression of *Sox2* in G-type and GI-mixed-type stomach adenocarcinomas and suppression in the I type and N types. It remains to be elucidated whether gastric and intestinal gene transcription is regulated independently or in a coordinated fashion. This question and the possible existence of other essential cofactors clearly warrant further investigation.

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