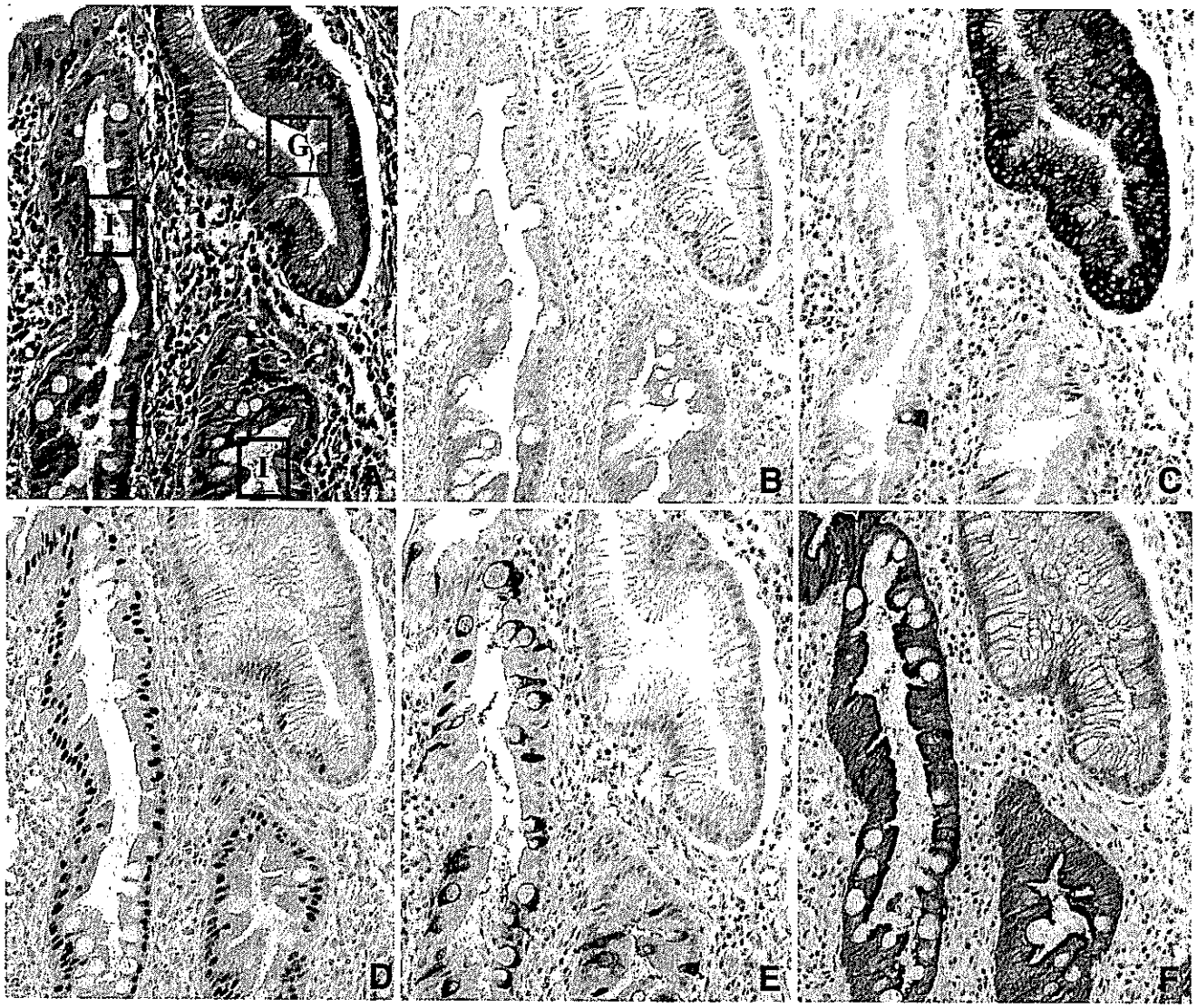

Fig. 1A–F. Immunohistochemical staining of normal human gastric mucosa and intestinal metaplasia. *G*, Pyloric gland with a gastric phenotype expressing Sox2 and MUC5AC; *I*, intestinal metaplastic gland harboring goblet cells producing Cdx2, MUC2, and villin. **A** H&E staining; **B–F** immunohistochemistry; 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

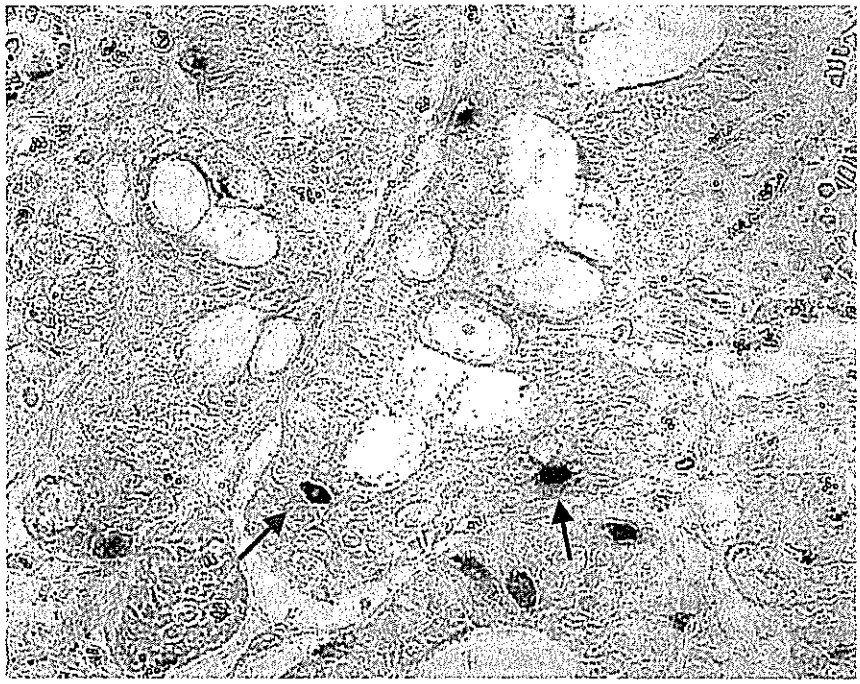

Fig. 2. An example of double staining of gastrin and glucagon-like peptide (GLP)-1 in gastric-and-intestinal mixed phenotype intestinal metaplasia (GI-IM). A mixture of gastrin- and GLP-1-positive endocrine cells is apparent in the same gland. Gastrin-positive cells (*blue arrow*) and GLP-1-positive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

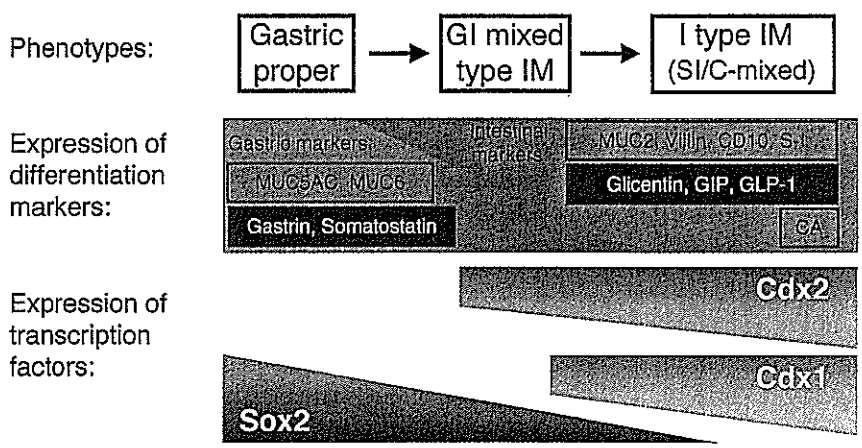

Fig. 3. Schematic view of progression of intestinal metaplasia (*IM*). Gastric mucosa changes to gastric-and-intestinal (*GI*) mixed-type IM, and then progresses to Solely intestinal (*I*)-type IM. Within the I type, small-intestinal (*SI*) type cells can colocalize with G phenotype cells, whereas colonic (*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[c] Proton pump beta subunit[c]
Enzymes		Pepsinogen II[c]		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[h]	
		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.

Acknowledgments The authors thank colleagues in their laboratory for their expert technical assistance and valuable discussion. This work was supported in part by a Grant-in-Aid for the Third-term Comprehensive 10-year Strategy for Cancer Control, and a Grant-in-Aid for Cancer Research, both from the Ministry of Health, Labour, and Welfare, Japan; and a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

References

1. Warren JR, Marshall BJ. Unidentified curved bacilli on gastric epithelium in active chronic gastritis. Lancet 1983;1:1273–5.
2. Forman D, Newell DG, Fullerton F, Yarnell JW, Stacey AR, Wald N, et al. Association between infection with *Helicobacter pylori* and risk of gastric cancer: evidence from a prospective investigation. BMJ 1991;302:1302–5.
3. Nomura A, Stemmermann GN, Chyou PH, Kato I, Perez-Perez GI, Blaser MJ. *Helicobacter pylori* infection and gastric carcinoma among Japanese Americans in Hawaii. N Engl J Med 1991;325:1132–6.
4. Parsonnet J, Friedman GD, Vandersteen DP, Chang Y, Vogelman JH, Orentreich N, et al. *Helicobacter pylori* infection and the risk of gastric carcinoma. N Engl J Med 1991;325:1127–31.
5. Uemura N, Okamoto S, Yamamoto S, Matsumura N, Yamaguchi S, Yamakido M, et al. *Helicobacter pylori* infection and the development of gastric cancer. N Engl J Med 2001;345:784–9.
6. Sepulveda AR, Coelho LG. *Helicobacter pylori* and gastric malignancies. Helicobacter 2002;7(Suppl 1):37–42.
7. Walker MM. Is intestinal metaplasia of the stomach reversible? Gut 2003;52:1–4.
8. IARC Working Group on the Evaluation of Carcinogenic Risks to Humans: Infection with *Helicobacter pylori*. In: Schistosomes, Liver Flukes and *Helicobacter pylori*, IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, Vol. 61. World Health Organization/International Agency for Research on Cancer, Lyon, 1994, pp. 177–241.
9. Kawachi T, Kurisu M, Numanyu N, Sasajima K, Sano T. Precancerous changes in the stomach. Cancer Res 1976;36:2673–7.
10. Matsukura N, Suzuki K, Kawachi T, Aoyagi M, Sugimura T, Kitaoka H, et al. Distribution of marker enzymes and mucin in intestinal metaplasia in human stomach and relation to complete and incomplete types of intestinal metaplasia to minute gastric carcinomas. J Natl Cancer Inst 1980;65:231–40.

11. Morson BC. Carcinoma arising from areas of intestinal metaplasia in the gastric mucosa. Br J Cancer 1955;9:377–85.
12. Filipe MI, Ramachandra S. The histochemistry of intestinal mucins: changes in disease. In: Whitehead R, editor. Gastrointestinal and oesophageal pathology. Edinburgh: Churchill Livingstone; 1995. p. 73–95.
13. Filipe MI, Potet F, Bogomoletz WV, Dawson PA, Fabiani B, Chauveinc P, et al. Incomplete sulphomucin-secreting intestinal metaplasia for gastric cancer. Preliminary data from a prospective study from three centres. Gut 1985;26:1319–26.
14. Filipe MI, Munoz N, Matko I, Kato I, Pompe-Kirn V, Jutersek A, et al. Intestinal metaplasia types and the risk of gastric cancer: a cohort study in Slovenia. Int J Cancer 1994;57:324–9.
15. Matsukuma A, Mori M, Enjoji M. Sulphomucin-secreting intestinal metaplasia in the human gastric mucosa. An association with intestinal-type gastric carcinoma. Cancer 1990;66:689–94.
16. Rokkas T, Filipe MI, Sladen GE. Detection of an increased incidence of early gastric cancer in patients with intestinal metaplasia type III who are closely followed up. Gut 1991;32:1110–3.
17. Silva S, Filipe MI. Intestinal metaplasia and its variants in the gastric mucosa of Portuguese subjects: a comparative analysis of biopsy and gastrectomy material. Hum Pathol 1986;17:988–95.
18. You WC, Blot WJ, Li JY, Chang YS, Jin ML, Kneller R, et al. Precancerous gastric lesions in a population at high risk of stomach cancer. Cancer Res 1993;53:1317–21.
19. Stemmermann GN. Intestinal metaplasia of the stomach. A status report. Cancer 1994;74:556–64.
20. Kato Y, Kitagawa T, Yanagisawa A, Kubo K, Utsude T, Hiratsuka H, et al. Site-dependent development of complete and incomplete intestinal metaplasia types in the human stomach. Jpn J Cancer Res 1992;83:178–83.
21. Lauren P. The two histological main types of gastric carcinoma: diffuse and so-called intestinal-type carcinoma: an attempt at a histo-clinical classification. Acta Pathol Microbiol Scand 1965;64: 31–49.
22. Yuasa Y. Control of gut differentiation and intestinal-type gastric carcinogenesis. Nat Rev Cancer 2003;3:592–600.
23. Kusakabe M, Yokoyama M, Sakakura T, Nomura T, Hosick HL, Nishizuka Y. A novel methodology for analysis of cell distribution in chimeric mouse organs using a strain specific antibody. J Cell Biol 1988;107:257–65.
24. Tatematsu M, Fukami H, Yamamoto M, Nakanishi H, Masui T, Kusakabe N, et al. Clonal analysis of glandular stomach carcinogenesis in C3H/HeN(-)BALB/c chimeric mice treated with N-methyl-N-nitrosourea. Cancer Lett 1994;83:37–42.
25. Tatematsu M, Masui T, Fukami H, Yamamoto M, Nakanishi H, Inada K, et al. Primary monoclonal and secondary polyclonal growth of colon neoplastic lesions in C3H/HeN(-)BALB/c chimeric mice treated with 1,2-dimethylhydrazine: immunohistochemical detection of C3H strain-specific antigen and simple sequence length polymorphism analysis of DNA. Int J Cancer 1996;66:234–8.
26. Tsukamoto T, Yamamoto M, Fukami H, Yoshikawa A, Sakai H, Hirata A, et al. Susceptibility to colon carcinogenesis in C3H (–)C57BL/6 chimeric mice reflects both tissue microenvironment and genotype. Cancer Lett 2006 (in press).
27. Fialkow PJ. Clonal origin of human tumors. Biochim Biophys Acta 1976;458:283–321.
28. Kakizoe T, Tanooka H, Tanaka K, Sugimura T. Single-cell origin of bladder cancer induced by N-butyl-N-(4-hydroxybutyl) nitrosamine in mice with cellular mosaicism. Gann 1983;74: 462–5.
29. Nomura S, Kaminishi M, Sugiyama K, Oohara T, Esumi H. Clonal analysis of isolated intestinal metaplastic glands of stomach using X-linked polymorphism. Gut 1998;42:663–8.
30. Winton DJ, Blount MA, Ponder BA. A clonal marker induced by mutation in mouse intestinal epithelium. Nature 1988;333:463–6.
31. Park HS, Goodlad RA, Wright NA. Crypt fission in the small intestine and colon. A mechanism for the emergence of G6PD locus-mutated crypts after treatment with mutagens. Am J Pathol 1995;147:1416–27.
32. Potten CS. Stem cells in gastrointestinal epithelium: numbers, characteristics and death. Philos Trans R Soc Lond B Biol Sci 1998;353:821–30.
33. Katsuyama T, Spicer SS. Histochemical differentiation of complex carbohydrates with variants of the concanavalin A-horseradish peroxidase method. J Histochem Cytochem 1978;26: 233–50.
34. Tatematsu M, Katsuyama T, Fukushima S, Takahashi M, Shirai T, Ito N, et al. Mucin histochemistry by paradoxical concanavalin A staining in experimental gastric cancers induced in Wistar rats by N-methyl-N-nitro-N-nitrosoguanidine or 4-nitroquinoline 1-oxide. J Natl Cancer Inst 1980;64:835–43.
35. Furihata C, Tatematsu M, Miki K, Katsuyama T, Sudo K, Miyagi N, et al. Gastric- and intestinal-type properties of human gastric cancers transplanted into nude mice. Cancer Res 1984;44:727–33.
36. Tatematsu M, Furihata C, Katsuyama T, Mera Y, Inoue T, Matsushima T, et al. Immunohistochemical demonstration of pyloric gland-type cells with low-pepsinogen isozyme 1 in preneoplastic and neoplastic tissues of rat stomachs treated with N-methyl-N′-nitro-N-nitrosoguanidine. J Natl Cancer Inst 1987; 78:771–7.
37. Tatematsu M, Katsuyama T, Furihata C, Fukushima S, Shirai T, Kato T, et al. Cellular differentiation and histogenesis of rat glandular stomach cancers. Jpn J Cancer Res 1990;81:760–7.
38. Iwata H, Itzkowitz SH, Werther JL, Hayashi K, Nakamura H, Ichinose M, et al. Expression of sialosyl-Tn in intestinal type cancer cells of human gastric cancers. Acta Pathol Jpn 1993;43: 646–53.
39. Inada K, Nakanishi H, Fujimitsu Y, Shimizu N, Ichinose M, Miki K, et al. Gastric and intestinal mixed and solely intestinal types of intestinal metaplasia in the human stomach. Pathol Int 1997;47: 831–41.
40. Inada K, Tanaka H, Nakanishi H, Tsukamoto T, Ikehara Y, Tatematsu K, et al. Identification of Paneth cells in pyloric glands associated with gastric and intestinal mixed-type intestinal metaplasia of the human stomach. Virchows Arch 2001;439:14–20.
41. Tajima Y, Shimoda T, Nakanishi Y, Yokoyama N, Tanaka T, Shimizu K, et al. Gastric and intestinal phenotypic marker expression in gastric carcinomas and its prognostic significance: immunohistochemical analysis of 136 lesions. Oncology 2001;61:212–20.
42. Kawachi T, Kogure K, Tanaka N, Tokunaga A, Sugimura T. Studies of intestinal metaplasia in the gastric mucosa by detection of disaccharidases with "Tes-Tape". J Natl Cancer Inst 1974;53: 19–30.
43. Teglbjaerg PS, Nielsen HO. "Small intestinal type" and "colonic type" intestinal metaplasia of the human stomach, and their relationship to the histogenetic types of gastric adenocarcinoma. Acta Pathol Microbiol Scand [A] 1978;86A:351–5.
44. Segura DI, Montero C. Histochemical characterization of different types of intestinal metaplasia in gastric mucosa. Cancer 1983; 52:498–503.
45. Jass JR, Filipe MI. A variant of intestinal metaplasia associated with gastric carcinoma: a histochemical study. Histopathology 1979;3:191–9.
46. Karam SM, Leblond CP. Dynamics of epithelial cells in the corpus of the mouse stomach. I. Identification of proliferative cell types and pinpointing of the stem cell. Anat Rec 1993;236:259–79.
47. Ponder BA, Schmidt GH, Wilkinson MM, Wood MJ, Monk M, Reid A. Derivation of mouse intestinal crypts from single progenitor cells. Nature 1985;313:689–91.
48. Hattori T, Fujita S. Tritiated thymidine autoradiographic study of cell migration and renewal in the pyloric mucosa of golden hamsters. Cell Tissue Res 1976;175:49–57.
49. Yuasa H, Inada K, Watanabe H, Tatematsu M. A phenotypic shift from gastric-intestinal to solely intestinal cell types in intestinal metaplasia in rat stomach following treatment with X-rays. J Toxicol Pathol 2002;15:85–93.

50. Nozaki K, Shimizu N, Tsukamoto T, Inada K, Cao X, Ikehara Y, et al. Reversibility of heterotopic proliferative glands in glandular stomach of *Helicobacter pylori*-infected Mongolian gerbils on eradication. Jpn J Cancer Res 2002;93:374–81.
51. Yamamoto M, Furihata C, Ogiu T, Tsukamoto T, Inada K, Hirano K, et al. Independent variation in susceptibilities of six different mouse strains to induction of pepsinogen-altered pyloric glands and gastric tumor intestinalization by N-methyl-N-nitrosourea. Cancer Lett 2002;179:121–32.
52. Mallo GV, Rechreche H, Frigerio JM, Rocha D, Zweibaum A, Lacasa M, et al. Molecular cloning, sequencing and expression of the mRNA encoding human Cdx1 and Cdx2 homeobox. Down-regulation of Cdx1 and Cdx2 mRNA expression during colorectal carcinogenesis. Int J Cancer 1997;74:35–44.
53. Silberg DG, Furth EE, Taylor JK, Schuck T, Chiou T, Traber PG. CDX1 protein expression in normal, metaplastic, and neoplastic human alimentary tract epithelium. Gastroenterology 1997;113: 478–86.
54. Mizoshita T, Inada K, Tsukamoto T, Kodera Y, Yamamura Y, Hirai T, et al. Expression of Cdx1 and Cdx2 mRNAs and relevance of this expression to differentiation in human gastrointestinal mucosa — with special emphasis on participation in intestinal metaplasia of the human stomach. Gastric Cancer 2001;4:185–91.
55. Eda A, Osawa H, Yanaka I, Satoh K, Mutoh H, Kihira K, et al. Expression of homeobox gene CDX2 precedes that of CDX1 during the progression of intestinal metaplasia. J Gastroenterol 2002;37:94–100.
56. Satoh K, Mutoh H, Eda A, Yanaka I, Osawa H, Honda S, et al. Aberrant expression of CDX2 in the gastric mucosa with and without intestinal metaplasia: effect of eradication of *Helicobacter pylori*. Helicobacter 2002;7:192–8.
57. Drummond F, Sowden J, Morrison K, Edwards YH. The caudal-type homeobox protein Cdx-2 binds to the colon promoter of the carbonic anhydrase 1 gene. Eur J Biochem 1996;236:670–81.
58. Suh E, Chen L, Taylor J, Traber PG. A homeodomain protein related to caudal regulates intestine-specific gene transcription. Mol Cell Biol 1994;14:7340–51.
59. Troelsen JT, Mitchelmore C, Spodsberg N, Jensen AM, Noren O, Sjostrom H. Regulation of lactase-phlorizin hydrolase gene expression by the caudal-related homoeodomain protein Cdx-2. Biochem J 1997;322(Pt 3):833–8.
60. Park J, Schulz S, Waldman SA. Intestine-specific activity of the human guanylyl cyclase C promoter is regulated by Cdx2. Gastroenterology 2000;119:89–96.
61. Yamamoto H, Bai YQ, Yuasa Y. Homeodomain protein CDX2 regulates goblet-specific MUC2 gene expression. Biochem Biophys Res Commun 2003;300:813–8.
62. Subramanian V, Meyer B, Evans GS. The murine Cdx1 gene product localises to the proliferative compartment in the developing and regenerating intestinal epithelium. Differentiation 1998; 64:11–8.
63. Lickert H, Domon C, Huls G, Wehrle C, Duluc I, Clevers H, et al. Wnt/beta-catenin signaling regulates the expression of the homeobox gene Cdx1 in embryonic intestine. Development 2000;127:3805–13.
64. Domon-Dell C, Freund JN. Stimulation of Cdx1 by oncogenic beta-catenin/Tcf4 in colon cancer cells; opposite effect of the CDX2 homeoprotein. FEBS Lett 2002;518:83–7.
65. Yuasa Y, Nagasaki H, Akiyama Y, Sakai H, Nakajima T, Ohkura Y, et al. Relationship between CDX2 gene methylation and dietary factors in gastric cancer patients. Carcinogenesis 2005;26: 193–200.
66. Bowles J, Schepers G, Koopman P. Phylogeny of the SOX family of developmental transcription factors based on sequence and structural indicators. Dev Biol 2000;227:239–55.
67. Wood HB, Episkopou V. Comparative expression of the mouse Sox1, Sox2 and Sox3 genes from pre-gastrulation to early somite stages. Mech Dev 1999;86:197–201.
68. Ishii Y, Rex M, Scotting PJ, Yasugi S. Region-specific expression of chicken Sox2 in the developing gut and lung epithelium: regulation by epithelial-mesenchymal interactions. Dev Dyn 1998;213: 464–75.
69. Sakamoto N, Fukuda K, Watanuki K, Sakai D, Komano T, Scotting PJ, et al. Role for cGATA-5 in transcriptional regulation of the embryonic chicken pepsinogen gene by epithelial-mesenchymal interactions in the developing chicken stomach. Dev Biol 2000;223:103–13.
70. Tsukamoto T, Inada K, Tanaka H, Mizoshita T, Mihara M, Ushijima T, et al. Down regulation of a gastric transcription factor, Sox2, and ectopic expression of intestinal homeobox genes, Cdx1 and Cdx2: inverse correlation during progression from gastric/intestinal-mixed to complete intestinal metaplasia. J Cancer Res Clin Oncol 2004;130:135–45.
71. Li XL, Eishi Y, Bai YQ, Sakai H, Akiyama Y, Tani M, et al. Expression of the SRY-related HMG box protein SOX2 in human gastric carcinoma. Int J Oncol 2004;24:257–63.
72. Tsukamoto T, Mizoshita T, Mihara M, Tanaka H, Takenaka Y, Yamamura Y, et al. Sox2 expression in human stomach adenocarcinomas with gastric and gastric-and-intestinal-mixed phenotypes. Histopathology 2005;46:649–58.
73. Sakai H, Eishi Y, Li XL, Akiyama Y, Miyake S, Takizawa T, et al. PDX1 homeobox protein expression in pseudopyloric glands and gastric carcinomas. Gut 2004;53:323–30.
74. Faller G, Kirchner T. Immunological and morphogenic basis of gastric mucosa atrophy and metaplasia. Virchows Arch 2005;446: 1–9.
75. Verrijzer CP, Van der Vliet PC. POU domain transcription factors. Biochim Biophys Acta 1993;1173:1–21.
76. Jin T, Li H. Pou homeodomain protein OCT1 is implicated in the expression of the caudal-related homeobox gene Cdx-2. J Biol Chem 2001;276:14752–8.
77. van den Brink GR, Hardwick JC, Nielsen C, Xu C, ten Kate FJ, Glickman J, et al. Sonic hedgehog expression correlates with fundic gland differentiation in the adult gastrointestinal tract. Gut 2002;51:628–33.
78. Kim JH, Huang Z, Mo R. Gli3 null mice display glandular overgrowth of the developing stomach. Dev Dyn 2005;234:984–91.
79. Lund AH, van Lohuizen M. RUNX: a trilogy of cancer genes. Cancer Cell 2002;1:213–5.
80. Li QL, Ito K, Sakakura C, Fukamachi H, Inoue K, Chi XZ, et al. Causal relationship between the loss of RUNX3 expression and gastric cancer. Cell 2002;109:113–24.
81. Kim TY, Lee HJ, Hwang KS, Lee M, Kim JW, Bang YJ, et al. Methylation of RUNX3 in various types of human cancers and premalignant stages of gastric carcinoma. Lab Invest 2004;84:479–84.
82. Osaki M, Moriyama M, Adachi K, Nakada C, Takeda A, Inoue Y, et al. Expression of RUNX3 protein in human gastric mucosa, intestinal metaplasia and carcinoma. Eur J Clin Invest 2004;34: 605–12.
83. Fukamachi H, Ito K, Ito Y. Runx3-/- gastric epithelial cells differentiate into intestinal type cells. Biochem Biophys Res Commun 2004;321:58–64.
84. Tanaka H, Tsukamoto T, Mizoshita T, Inada K, Ogasawara N, Cao X, et al. Expression of small intestinal and colonic phenotypes in complete intestinal metaplasia of the human stomach. Virchows Arch 2005;447:806–15.

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

Xueyuan Cao,[1] Tetsuya Tsukamoto,[1,4] Koji Nozaki,[1] Harunari Tanaka,[1] Liyu Cao,[1,2] Takeshi Toyoda,[1] Shinji Takasu,[1] Hisayo Ban[1] Toshiko Kumagai[3] and Masae Tatematsu[1]

[1]Division of Oncological Pathology, Aichi Cancer Center Research Institute, 1-1 Kanokoden, Chikusa-ku, Nagoya, Aichi 464-8681;[2]Department of Pathology, Anhui Medical University, Hefei, China;[3]Central Clinical Laboratories, Shinshu University Hospital, 3-1-1 Asahi, Matsumoto 390-8621

(Received September 19, 2006/Revised November 19, 2006/Accepted December 11, 2006/Online publication January 26, 2007)

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.

[4]To whom requests for reprints should be addressed. E-mail: ttsukamt@aichi-cc.jp

doi: 10.1111/j.1349-7006.2007.00416.x

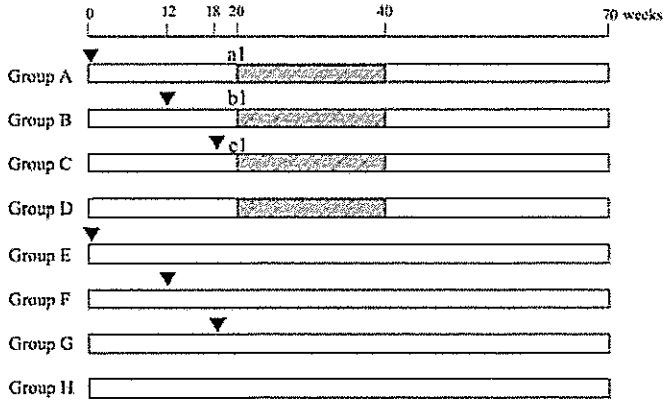

Fig. 1. Experimental design. Animals, 7-week-old male Mongolian gerbils, ▼, *H. pylori* (i.g.), ■, *N*-methyl-*N*-nitorosourea in drinking water at the concentration of 10 p.p.m.

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

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

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).

doi: 10.1111/j.1349-7006.2007.00416.x

Table 2. Histopathological response in gerbils

Group	No.	Antrum						Corpus						Anti-*Hp* IgG titer (A.I)	Serum gastin levels (pg/mL)
		Mucosal thickness (mm)	Neutrophils	Mono-nuclear cells	Hyperplasia	Intestinal metaplasia	BrdU labeling index (%)	Mucosal thickness (mm)	Neutrophils	Mono-nuclear cells	Hyperplasia	Intestinal metaplasia	BrdU labeling index (%)		
A	20	0.82 ± 0.25*	2.1 ± 0.6*	3.0 ± 0.2*	2.9 ± 0.3*	2.3 ± 0.7*	24.6 ± 5.9*	1.04 ± 0.26*	1.3 ± 0.3*	2.4 ± 0.3*	2.4 ± 0.2*	1.1 ± 0.4*	14.1 ± 3.3*	288.1 ± 44.7*	1235.1 ± 373.1*
a1	6	0.35 ± 0.09[†]	2.9 ± 0.2[‡]	3.0 ± 0.0[‡]	1.3 ± 0.3[†]	0.0 ± 0.0	20.2 ± 6.3[‡]	0.64 ± 0.07[†]	1.0 ± 0.1[†]	1.0 ± 0.3[‡]	1.1 ± 0.2[†]	0.0 ± 0.0	4.5 ± 1.5	14.3 ± 3.5[‡]	ND
B	10	0.48 ± 0.20	1.7 ± 0.3	2.3 ± 0.2	1.8 ± 0.6	0.7 ± 0.4	13.4 ± 4.2	0.77 ± 0.29	1.2 ± 0.3	1.6 ± 0.6	1.6 ± 0.5	0.3 ± 0.3	9.6 ± 2.1	175.8 ± 23.1	323.6 ± 114.4
b1	7	0.30 ± 0.11	2.4 ± 0.2	2.4 ± 0.4	1.4 ± 0.2	0.0 ± 0.0	10.4 ± 3.8	0.54 ± 0.06	1.0 ± 0.0	1.1 ± 0.2	1.1 ± 0.3	0.0 ± 0.0	4.4 ± 1.0	8.8 ± 1.4	ND
C	13	0.60 ± 0.17	1.4 ± 0.3	2.1 ± 0.3	1.8 ± 0.3	0.6 ± 0.2	8.3 ± 1.8	0.77 ± 0.21	1.1 ± 0.0	1.5 ± 0.4	1.4 ± 0.2	0.5 ± 0.4	9.3 ± 2.3	137.1 ± 19.6	395.0 ± 68.3
c1	7	0.19 ± 0.02	1.0 ± 0.1	1.0 ± 0.1	0.7 ± 0.3	0.0 ± 0.0	7.2 ± 1.8	0.45 ± 0.08	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.0 ± 0.0	3.5 ± 1.3	0.8 ± 0.3	ND
D	16	0.20 ± 0.04	0.0 ± 0.0	0.4 ± 0.1	0.0 ± 0.0	0.0 ± 0.1	4.4 ± 1.9	0.42 ± 0.04	0.0 ± 0.0	0.3 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	3.0 ± 0.8	1.4 ± 0.3	206.2 ± 18.6
E	5	0.62 ± 0.13[§]	1.9 ± 0.2[¶]	3.0 ± 0.0[¶]	2.8 ± 0.3[¶]	2.7 ± 0.3[§]	20.4 ± 4.8[§]	1.02 ± 0.18[¶]	1.5 ± 0.4[¶]	2.4 ± 0.2[¶]	2.0 ± 0.4[¶]	1.1 ± 0.4[¶]	13.7 ± 1.6[§]	498.4 ± 74.0[§]	807.6 ± 117.0[§]
F	5	0.41 ± 0.09	1.6 ± 0.2	2.5 ± 0.4	2.3 ± 0.3	1.0 ± 0.0	10.2 ± 2.4	0.86 ± 0.05	1.2 ± 0.3	2.0 ± 0.0	1.7 ± 0.6	0.7 ± 0.3	5.9 ± 2.1	115.2 ± 36.5	396.3 ± 61.6
G	5	0.34 ± 0.08	1.3 ± 0.3	2.0 ± 0.0	1.9 ± 0.4	0.6 ± 0.2	4.5 ± 1.6	0.71 ± 0.22	1.0 ± 0.1	1.4 ± 0.2	1.0 ± 0.0	0.5 ± 0.1	3.9 ± 1.6	102.0 ± 37.5	375.2 ± 89.1
H	6	0.19 ± 0.03	0.0 ± 0.0	0.3 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	2.1 ± 0.4	0.44 ± 0.06	0.0 ± 0.0	0.3 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	2.7 ± 0.9	1.3 ± 0.4	244.7 ± 8.8

*$P < 0.05$ to group B and C; [†]$P < 0.05$ to group b1; [‡]$P < 0.05$ to group b1 and c1; [§]$P < 0.05$ to group F and G; [¶]$P < 0.05$ to group G; ND, not determined; *Hp*, *H.pylori*; BrdU, bromodeoxyuridine

Fig. 2. Histopathological findings of pyloric mucosa after inoculation of *H. pylori*. (a) Marked lymphoid follicle formation in a group a1 gerbil (H&E; × 25); (b) Mild gastritis in a group c1 gerbil (H&E; × 25); (c) Marked gastritis with heterotopic proliferative glands (HPGs) at 70 weeks post-infection (group A, H&E; × 25); (D) Moderate gastritis in a group C gerbil (H&E; × 25).

Fig. 3. Histology of adenocarcinomas. (a) Well differentiated adenocarcinoma in glandular stomach at 70 weeks post-*H. pylori* infection in a group A gerbil (H&E; × 80). (b) Signet ring-cell carcinoma at 70 weeks post-infection in a group A gerbil (H&E; × 400).

Expression of cytokine mRNAs in gastric mucosa of gerbils. IL-1β, TNF-α, COX-2 and iNOS mRNA levels were very low in uninfected gerbils at 77 weeks of age. Infection with the H. pylori resulted in significant increase in transcripts at 20 and 70 weeks in long-term infection gerbils compared with middle and short-term infection gerbils (Fig. 4). The mRNA levels at 70 week post-infection were greater than at 20 weeks post-infection. In H. pylori-infected gerbils, IL-1β, TNF-α, COX-2, and iNOS mRNA levels correlated significantly with chronic active inflammation scores in the gastric mucosa.

Anti-*H. pylori* IgG titers and gastrin levels. The serum anti-H. pylori IgG titers and gastrin levels were significantly higher in the long-term infection group than in the middle and short-term groups (Table 2).

Discussion

At 20 and 70 weeks post-infection, histologic examination revealed more severe neutrophil and mononuclear cells infiltration and

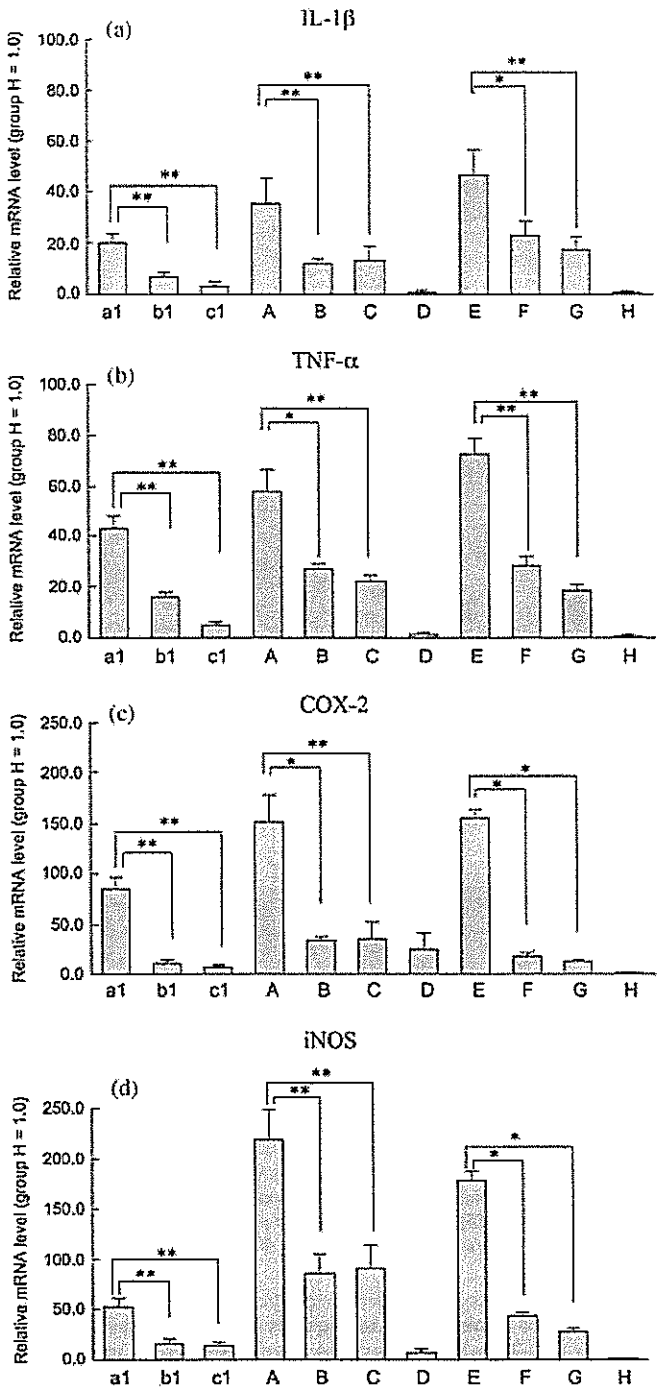

Fig. 4. Relative expression levels of IL-1β, TNF-α, COX-2 and iNOS mRNAs in glandular stomachs of gerbils. (a) IL-1β; (b) TNFα; (c) COX-2; (d) iNOS. Values were set at 1.0 in group H and expressed as mean ± SE relative values. Note increase in relative IL-1β, TNFα, COX-2 and iNOS mRNA expression levels in group A as compared to groups B and C; in group a1 as compared to groups b1 and c1; in group E as compared to groups F and G. $*P < 0.05$ and $**P < 0.01$, by the Bonferroni multiple-comparison test.

submucosal lymphoid follicle formation in the antrum mucosa in early/long-term groups than in the middle and short-term H. pylori-infected groups, and this correlated with the yield of adenocarcinomas. The results thus provide support for the conclusion that infiltration of polymorphonuclear neutrophils and mucosa-associated lymphoid tissue, as well as damage to the epithelial cells, characterizes the first phase of gastric carcinogenesis in this model.[20] Neutrophil infiltration is an almost invariable finding in H. pylori-associated gastritis and is topographically related to H. pylori colonization. It is now evident that inflammatory cells have powerful effects on gastric mucosal injury, neutrophils being recruited to the gastric mucosa and generate reactive oxygen and nitrogen species and proteases.[21] However, neutrophils are not able to kill the H. pylori that live in the gastric mucus, and compounds produced by activated neutrophils themselves are potentially harmful for normal tissue. Active neutrophil infiltration in gastric H. pylori infection may contribute to the increased levels of mutation also observed in Big Blue transgenic mice.[22]

The type of host immune response against H. pylori is considered crucial for the outcome of the infection.[23] One possible mechanism involves up-regulation of inflammatory responses through increase of synthesis and release of pro-inflammatory mediators, such as iNOS and COX-2, via effects on nuclear factor κB (NF-κB).[24,25] A predominant H. pylori-specific Th1 response, characterized by high TNF-α, IL-1β and interferon-γ (IFN-γ) production is associated with gastritis and peptic ulcer.[26] We found mRNA levels of mucosal IL-1β and TNF-α mRNA levels were significantly higher in gerbils with long-term H. pylori-infection than in those with middle and the short-term infection. Yamaoka et al. reported that IL-1β and IFN-γ play important roles in the acute phase of pyloric inflammation in H. pylori-infected gerbils.[12] H. pylori stimulates NF-κB activation and chemokine interleukin-8 (IL-8) expression in gastric epithelial cells. Epithelial IL-8 can be induced not only by direct stimulation of H. pylori, but also following exposure to the endogenous proinflammatory mediators IL-1 and TNF-α in the gastric mucosa.[27,28]

COX-2 and iNOS may play important roles in stomach tumor growth and progression. COX-2 is frequently undetectable in normal tissues but is induced by cytokines, growth factors, reactive oxygen species and tumor promoters,[29,30] and catalyzes the committed step in the conversion of arachidonic acid to protumorigenic eicosanoids, such as prostaglandin E2, which are involved in the maintenance of tumor integrity.[30,31] Several reports have documented H. pylori up-regulation of COX-2 mRNA expression and release of prostaglandin E2 from a gastric cancer cell line, as well as in the gastric mucosa of gerbil models and in humans.[32–34] In the present study, the fact that long-term H. pylori infection significantly up-regulated COX-2 expression is of clear interest.

A number of previous findings also suggest that iNOS contributes to H pylori-associated gastric carcinogenesis in mice and man. Increased iNOS activity has been observed in patients with chronic gastritis and gastric adenocarcinoma patients,[35] nitric oxide (NO) endogenously produced by this family of enzymes causing mutations and DNA deamination.[36,37] Our present observations suggest that COX-2 and iNOS are both induced in H. pylori-positive gastritis and thus may modulate the inflammation and alterations in epithelial cell growth that occur with this disease. The increase in proliferative activity in the mucosa is another feature of interest. At 20 and 70 weeks post-infection, BrdU-labeled cells represented almost 20–25% of the antrum epithelial cells in long-term infected gerbils (groups a1 and A). Early inoculation and long-term H. pylori infection thus appeared to increase epithelial cell apoptosis and proliferation, and this would be expected to lead to increased susceptibility to carcinogens.

Immunohistochemical study showed increased expression of iNOS and COX-2 genes in H. pylori gastritis in humans: iNOS protein was detected in epithelium, endothelium, and lamina propria inflammatory cells, and COX-2 protein localized to mononuclear and fibroblast cells in the lamina propria.[24] Thus, iNOS as well as other inflammatory cytokines may be expressed

doi: 10.1111/j.1349-7006.2007.00416.x

Table 3. Incidence of gastric carcinoma

Groups	Age colonized	Duration of colonization	Age sacrificed	No. of animals	Carcinoma		
					Dif.	Undif.	Incidence (%)
A *Hp* 20 w + MNU	7 w	70 w	77 w	20	12	1	13 (65.0)*
a1 before MNU treatment	7 w	20 w	27 w	6	0	0	0
B *Hp* 8 w + MNU	19 w	58 w	77 w	10	2	0	2 (20.0)
b1 before MNU treatment	19 w	8 w	27 w	7	0	0	0
C *Hp* 2 w + MNU	25 w	52 w	77 w	13	2	1	3 (23.1)
c1 before MNU treatment	25 w	2 w	27 w	7	0	0	0
D MNU	(-)	(-)	77 w	16	0	0	0
E *Hp* 70 w	7 w	70 w	77 w	5	0	0	0
F *Hp* 58 w	19 w	58 w	77 w	5	0	0	0
G *Hp* 52 w	25 w	52 w	77 w	5	0	0	0
H Control	(-)	(-)	77 w	6	0	0	0

*$P < 0.05$ between groups A, B and C, $P < 0.05$ to group B and group C, $P < 0.001$ to group D, by Fisher's exact test. *Hp*, *H. pylori* infection (i.g.); Dif, differentiated adenocarcinoma; Undif, undifferentiated adenocarcinoma. MNU, *N*-methyl-*N*-nitorosourea.

not only in inflammatory cells but also in epithelial cells in the H. pylori-infected Mongolian gerbils.

Group A showed lower anti-H. pylori IgG titer than that of the MNU untreated group (group E), although not statistically significant ($P < 0.08$). This tendency was not consistent between H. pylori + MNU and H. pylori alone groups (Groups B vs. F; Groups C vs. G) in anti-H. pylori IgG titers and gastrin levels (Table 2). Thus, the effects of MNU on mucosal atrophy could not be considered significant.

Of note, gerbils infected with H. pylori developed an antral-predominant gastritis, which progressed to corpus gastritis at 70 weeks. This pattern is typical of what is seen in humans living in regions of high gastric cancer incidence, and explains why H. pylori-related gastric cancer in gerbils develops most often in the antrum.[38] In the present study, we examined the inflammation status at two experimental timing points: 20 and 70 weeks. Among the 20-week groups (Groups a1, b1, and c1), Group a1 showed the highest inflammatory and proliferative responses, which might affect MNU-induced tumorigenesis. Thus, it could be concluded that the severity of H. pylori-induced inflammation may play important roles in gastric carcinogenesis.

Long-term H. pylori infection was here accompanied by significantly increased anti-H. pylori serum IgG antibody titer, highlighting the importance of the immune response of the host in the development of H. pylori-related gastric lesions. The results also suggest that the anti-H. pylori serum IgG antibody titer may be used as a marker of the severity of the H. pylori-infected chronic active gastritis.[18] A previous study demonstrated that acid secretion is decreased in gerbils infected with H. pylori.[39] In the present case, long-term H. pylori infection induced chronic pangastritis and may have suppressed parietal cell function, causing compensatory gastrin release corresponding with a lower gastric acid secretion. Furthermore, increased gastrin levels could contribute to gastric barrier dysfunction in H. pylori infection.[40] Wang et al. found that chronic hypergastrinemia can synergize with H. pylori infection and contribute to eventual parietal cell loss and progression to gastric cancer in insulin-gastrin (INS-GAS) transgenic mice.[41]

In summary, our study shows that the severity of chronic gastritis induced by pathogen H. pylori is linked with glandular gastric carcinogenesis. The gastric mucosal injury demonstrated in the long-standing H. pylori-infected gerbil clearly can lead to increased susceptibility to carcinogenic substances and also contribute to immune responses, perpetuation of mucosal inflammation and cancer development. This phenomenon is not only dependent on the timing and age at H. pylori infection, but also the period of infection.[42] The pathological changes present at the early stage of H. pylori infection seems to persist and become aggravated during the life span of the animals, thus contributing to the multifactorial processes underlying gastric neoplasia.

Acknowledgments

This work was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Health, Labor and Welfare, Japan, and a Grant-in-Aid from the Ministry of Education, Culture, Science, Sports and Technology of Japan. X. C. was a recipient of a postdoctoral fellowship from the Japan Society for the Promotion of Science during the performance of this research.

References

1 Kuipers EJ, Uyterlinde AM, Pena AS et al. Long-term sequelae of Helicobacter pylori gastritis. Lancet 1995; 345: 1525–8.
2 Parsonnet J, Friedman GD, Vandersteen DP et al. Helicobacter pylori infection and the risk of gastric carcinoma. N Engl J Med 1991; 325: 1127–31.
3 Coussens LM, Werb Z. Inflammation and cancer. Nature 2002; 420: 860–7.
4 Yoshida N, Granger DN, Evans DJ Jr. et al. Mechanisms involved in Helicobacter pylori-induced inflammation. Gastroenterology 1993; 105: 1431–40.
5 Yoshikawa T, Naito Y. The role of neutrophils and inflammation in gastric mucosal injury. Free Radic Res 2000; 33: 785–94.
6 Huang JQ, Sridhar S, Chen Y, Hunt RH. Meta-analysis of the relationship between Helicobacter pylori seropositivity and gastric cancer. Gastroenterology 1998; 114: 1169–79.
7 Uemura N, Okamoto S, Yamamoto S et al. Helicobacter pylori infection and the development of gastric cancer. N Engl J Med 2001; 345: 784–9.
8 Rad R, Brenner L, Bauer S et al. CD25(+) /Foxp3(+) T cells regulate gastric inflammation and Helicobacter pylori colonization in vivo. Gastroenterology 2006; 131: 525–37.
9 Hirayama F, Takagi S, Yokoyama Y, Iwao E, Ikeda Y. Establishment of gastric Helicobacter pylori infection in Mongolian gerbils. J Gastroenterol 1996; 31 (Suppl 9): 24–8.
10 Tatematsu M, Yamamoto M, Shimizu N et al. Induction of glandular stomach cancers in Helicobacter pylori-sensitive Mongolian gerbils treated with N-methyl-N-nitrosourea and N-methyl-N′-nitro-N-nitrosoguanidine in drinking water. Jpn J Cancer Res 1998; 89: 97–104.
11 Crabtree JE, Court M, Aboshkiwa MA, Jeremy AH, Dixon MF, Robinson PA. Gastric mucosal cytokine and epithelial cell responses to Helicobacter pylori infection in Mongolian gerbils. J Pathol 2004; 202: 197–207.
12 Yamaoka Y, Yamauchi K, Ota H et al. Natural history of gastric mucosal cytokine expression in Helicobacter pylori gastritis in Mongolian gerbils. Infect Immun 2005; 73: 2205–12.
13 Dixon MF, Genta RM, Yardley JH, Correa P. Classification and grading of gastritis. The updated sydney system. International workshop on the histopathology of gastritis, Houston 1994. Am J Surg Pathol 1996; 20: 1161–81.
14 Ikeno T, Ota H, Sugiyama A et al. Helicobacter pylori-induced chronic active gastritis, intestinal metaplasia, and gastric ulcer in Mongolian gerbils. Am J Pathol 1999; 154: 951–60.

15 Matsubara S, Shibata H, Takahashi M et al. Cloning of Mongolian gerbil cDNAs encoding inflammatory proteins, and their expression in glandular stomach during H. pylori infection. Cancer Sci 2004; 95: 798–802.
16 Otsuka T, Tsukamoto T, Tanaka H et al. Suppressive effects of fruit-juice concentrate of Prunus mume Sieb. et Zucc. (Japanese apricot, Ume) on Helicobacter pylori-induced glandular stomach lesions in Mongolian gerbils. Asian Pac J Cancer Prev 2005; 6: 337–41.
17 Tsukamoto T, Fukami H, Yamanaka S et al. Hexosaminidase-altered aberrant crypts, carrying decreased hexosaminidase alpha and beta subunit mRNAs, in colon of 1,2-dimethylhydrazine-treated rats. Jpn J Cancer Res 2001; 92: 109–18.
18 Kumagai T, Yan J, Graham DY et al. Serum immunoglobulin G immune response to Helicobacter pylori antigens in Mongolian gerbils. J Clin Microbiol 2001; 39: 1283–8.
19 Nozaki K, Shimizu N, Tsukamoto T et al. Reversibility of heterotopic proliferative glands in glandular stomach of Helicobacter pylori-infected Mongolian gerbils on eradication. Jpn J Cancer Res 2002; 93: 374–81.
20 Peek RM Jr, Crabtree JE. Helicobacter infection and gastric neoplasia. J Pathol 2006; 208: 233–48.
21 Siomek A, Rytarowska A, Szaflarska-Poplawska A et al. Helicobacter pylori infection is associated with oxidatively damaged DNA in human leukocytes and decreased level of urinary 8-oxo-7,8-dihydroguanine. Carcinogenesis 2006; 27: 405–8.
22 Jenks PJ, Jeremy AH, Robinson PA, Walker MM, Crabtree JE. Long-term infection with Helicobacter felis and inactivation of the tumour suppressor gene p53 cumulatively enhance the gastric mutation frequency in Big Blue transgenic mice. J Pathol 2003; 201: 596–602.
23 Roth KA, Kapadia SB, Martin SM, Lorenz RG. Cellular immune responses are essential for the development of Helicobacter felis-associated gastric pathology. J Immunol 1999; 163: 1490–7.
24 Fu S, Ramanujam KS, Wong A et al. Increased expression and cellular localization of inducible nitric oxide synthase and cyclooxygenase 2 in Helicobacter pylori gastritis. Gastroenterology 1999; 116: 1319–29.
25 Nozaki K, Tanaka H, Ikehara Y et al. Helicobacter pylori-dependent NF-kappa B activation in newly established Mongolian gerbil gastric cancer cell lines. Cancer Sci 2005; 96: 170–5.
26 D'Elios MM, Manghetti M, De Carli M et al. T helper 1 effector cells specific for Helicobacter pylori in the gastric antrum of patients with peptic ulcer disease. J Immunol 1997; 158: 962–7.
27 Keates S, Hitti YS, Upton M, Kelly CP. Helicobacter pylori infection activates NF-kappa B in gastric epithelial cells. Gastroenterology 1997; 113: 1099–109.
28 Yoshino N, Ishihara S, Rumi MA et al. Interleukin-8 regulates expression of Reg protein in Helicobacter pylori-infected gastric mucosa. Am J Gastroenterol 2005; 100: 2157–66.
29 Liu F, Pan K, Zhang X et al. Genetic variants in cyclooxygenase-2: expression and risk of gastric cancer and its precursors in a Chinese population. Gastroenterology 2006; 130: 1975–84.
30 Prescott SM, Fitzpatrick FA. Cyclooxygenase-2 and carcinogenesis. Biochim Biophys Acta 2000; 1470: M69–78.
31 Hansen-Petrik MB, McEntee MF, Jull B, Shi H, Zemel MB, Whelan J. Prostaglandin E(2) protects intestinal tumors from nonsteroidal anti-inflammatory drug-induced regression in Apc (Min/+) mice. Cancer Res 2002; 62: 403–8.
32 Ristimaki A, Honkanen N, Jankala H, Sipponen P, Harkonen M. Expression of cyclooxygenase-2 in human gastric carcinoma. Cancer Res 1997; 57: 1276–80.
33 Romano M, Ricci V, Memoli A et al. Helicobacter pylori up-regulates cyclooxygenase-2 mRNA expression and prostaglandin E2 synthesis in MKN 28 gastric mucosal cells in vitro. J Biol Chem 1998; 273: 28560–3.
34 Sakai T, Fukui H, Franceschi F et al. Cyclooxygenase expression during Helicobacter pylori infection in Mongolian gerbils. Dig Dis Sci 2003; 48: 2139–46.
35 Rajnakova A, Moochhala S, Goh PM, Ngoi S. Expression of nitric oxide synthase, cyclooxygenase, and p53 in different stages of human gastric cancer. Cancer Lett 2001; 172: 177–85.
36 Arroyo PL, Hatch-Pigott V, Mower HF, Cooney RV. Mutagenicity of nitric oxide and its inhibition by antioxidants. Mutat Res 1992; 281: 193–202.
37 Wink DA, Kasprzak KS, Maragos CM et al. DNA deaminating ability and genotoxicity of nitric oxide and its progenitors. Science 1991; 254: 1001–3.
38 Sepulveda A, Peterson LE, Shelton J, Gutierrez O, Graham DY. Histological patterns of gastritis in H. pylori-infected individuals with a family history of gastric cancer. Am J Gastroenterol 2002; 97: 1365–70.
39 Takashima M, Furuta T, Hanai H, Sugimura H, Kaneko E. Effects of Helicobacter pylori infection on gastric acid secretion and serum gastrin levels in Mongolian gerbils. Gut 2001; 48: 765–73.
40 Fukui H, Franceschi F, Penland RL et al. Effects of Helicobacter pylori infection on the link between regenerating gene expression and serum gastrin levels in Mongolian gerbils. Laboratory Invest 2003; 83: 1777–86.
41 Wang TC, Dangler CA, Chen D et al. Synergistic interaction between hypergastrinemia and Helicobacter infection in a mouse model of gastric cancer. Gastroenterology 2000; 118: 36–47.
42 Cao X, Tsukamoto T, Nozaki K et al. Earlier Helicobacter pylori infection increases the risk for the N-methyl-N-nitrosourea-induced stomach carcinogenesis in Mongolian gerbils. Jpn J Cancer Res 2002; 93: 1293–8.

doi: 10.1111/j.1349-7006.2007.00416.x

Histol Histopathol (2007) 22: 273-284

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Gastric and intestinal phenotypic correlation between exocrine and endocrine components in human stomach tumors

Y. Takenaka[1,2,*], T. Tsukamoto[1,*], T. Mizoshita[1], N. Ogasawara[1], N. Hirano[1], T. Otsuka[1], H. Ban[1], T. Nakamura[3], Y. Yamamura[4], M. Kaminishi[2] and M Tatematsu[1]

[1]Division of Oncological Pathology, Aichi Cancer Center Research Institute, Nagoya, Japan, [2]Department of Gastrointestinal Surgery, The University of Tokyo, Graduate School of Medicine, Tokyo, Japan, [3]Department of Gastroenterology, Aichi Cancer Center Hospital, Nagoya, Japan and [4]Department of Gastroenterological Surgery, Aichi Cancer Center Hospital, Japan

*These authors contributed equally to this work.

Summary. We have previously suggested that an origin of a stomach cancer is from a progenitor cell specializing toward exocrine cell (Exo-cell) lineages. To clarify whether our hypothesis is correct or not, we analyzed the expression of Exo-cell and endocrine cell (End-cell) markers in a series of lesions for comparison. We evaluated chromogranin A (CgA) expression in 37 early and 73 advanced stomach cancers, in 30 stomach adenomas, in 8 carcinoid tumors, and in 4 endocrine cell carcinomas (ECCs) with assessment of gastric and/or intestinal (G/I) phenotypes in both Exo-cell and End-cell by immunohistochemistry. CgA expression was observed in 10.8% of the early and 16.4% of the advanced stomach cancers, respectively. The End-cell G/I phenotypes were in line with the Exo-cell counterparts in the CgA-positive stomach cancerous areas, and there was strong association between Cdx2 expression and the intestinal End-cell markers. All of the adenoma cases had the intestinal Exo-cell phenotypic expression, with the positive link between Exo-cell and End-cell G/I phenotypes. All stomach carcinoids had CgA expression but no expression of Exo-cell markers. In conclusion, most stomach cancers might develop from a progenitor cell specializing towards Exo-cell lineages, but some cases possessed both Exo-cell and End-cell markers with maturely differentiated phenotypes. In such cases, Exo-cell and End-cell phenotypes were found to correlate strongly, suggesting the possibility of histogenesis from "cancer stem cells".

Offprint requests to: Tetsuya Tsukamoto, MD, Ph D, Division of Oncological Pathology, Aichi Cancer Centr Research Institute, 1-1 Kanokoden, Chikusa-ku, Nagoya 464-8681, Japan. e-mail: ttsukamt@aichi-cc.jp

Key words: Stomach cancers, Endocrine cell, Phenotypes, Progenitor cell, Cancer stem cells

Introduction

Gastrointestinal stem cells have the capacity for long-term self-replication and the ability to give rise to all other epithelial cell lineages (Schier and Wright, 2005). We have previously shown that each epithelial gland in the alimentary tract is derived from a single stem cell, based on clonality analysis using a strain specific antibody in C3H/HeN⇔BALB/c chimeric mice (Tatematsu et al., 1994, 1996; Tsukamoto et al., in press). The stem cell gives rise to two kinds of progenitor cell directly: (i) progenitor cell specializing toward exocrine cell (Exo-cell) lineages; and (ii) progenitor cells specializing toward endocrine cell (End-cell) lineages (Tatematsu et al., 2003, 2005).

Regarding the histogenesis of stomach cancer, if the cancer originated from the stem cell, the mixture of differentiation toward both Exo-cell and End-cell lineages should be observed more frequently and homogenously in the whole stomach cancerous tissues, although in fact it is observed rarely. Stomach epithelial tumors are divided into two major types: Exo-cell type (adenomas and carcinomas) and End-cell type [carcinoid tumors and endocrine cell carcinomas (ECC)]. Therefore, we have suggested the hypothesis that the origin of stomach cancers is from a progenitor cell specializing towards an Exo-cell lineage (Tatematsu et al., 2005). However, there have been several reports that chromogranin A (CgA), an End-cell differentiation marker, was immunohistochemically found in about 15-70% of human stomach cancers, although with

differences in anti-CgA antibodies and criteria of CgA positivity among the reports, suggesting the possession of both Exo-cell and End-cell differentiation in the stomach cancers (Park et al., 1992; Blumenfeld et al., 1996; Waldum et al., 1998; Qvigstad et al., 2000; Tzaneva, 2002; Naritomi et al., 2003). The existence of CgA positive stomach cancer cells indicates a concept that stomach cancer may occur from a stem cell harboring the ability to differentiate toward both Exo-cell and End-cell lineages. Recently, several reports have demonstrated the existence of malignant cells possessing self-renewal properties similar to normal stem cells in human myeloid leukemias (Bonnet and Dick, 1997), breast cancers (Al-Hajj et al., 2003) and brain tumors (Singh et al., 2004). There may be the possibility that the cancer stem cells appear secondarily in the stomach cancerous tissue, and they produce both Exo-cell and End-cell types, being similar to normal stem cells.

Human stomach cancers have been classified by Lauren into two major groups, the "intestinal" and "diffuse" types (Lauren, 1965), which respectively nearly correspond to the "differentiated" and "undifferentiated" types of Nakamura et al. (1968) and Sugano et al. (1982). 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 (Tatematsu et al., 2003). 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 intestinal epithelial cell markers such as MUC5AC, MUC6, MUC2, and villin (Tatematsu et al., 2003). In contrast, gastric and intestinal differentiation of endocrine cells in stomach cancers has not been fully evaluated.

To clarify whether our hypothesis is correct or not, the present study was conducted to analyze CgA expression by immunohistochemistry in a series of early and advanced stomach cancers with histological evaluation by hematoxylin and eosin (H&E) staining. The relations of gastric and intestinal differentiation between Exo- and End-cells were immunohistochemically evaluated within multiple areas within each stomach cancer case, and for comparison, adenoma cases and small numbers of carcinoids and ECCs, were similarly assessed.

Materials and methods

Samples and tissue collection

A total of 110 primary stomach cancers surgically resected at Aichi Cancer Center Hospital between 1994 and 2000 (Mizoshita et al., 2004a,b; Tsukamoto et al., 2005) were examined, 37 early and 73 advanced lesions, found in patients ranging in age from 43 to 78 years (mean±SD, 59.8±8.8 years) and 32 to 84 years (62.1±10.2 years), respectively. Histological classification was made into differentiated and undifferentiated adenocarcinomas according to the Japanese Classification of Gastric Carcinomas (Japanese Gastric Cancer Association, 1998). Early cases localized in mucosa (m) or in submucosa (sm). In the advanced cases, the cancers had invaded the muscularis propria (mp), the subserosa (ss), or the serosa and the peritoneal cavity (se), including adjacent organs (si).

We examined 12 primary endocrine tumors (8 carcinoids and 4 ECCs) surgically resected, too. The endocrine tumors were diagnosed by the presence of at least one of a number of endocrine markers, including CgA, synaptophysin, or CD56 and were found in 7 men and 5 women ranging in age from 39 to 66 years (52.0±10.8 years).

We also evaluated 30 stomach adenomas obtained by endoscopic mucosal resection or submucosal dissection. The stomach adenomas were found in 17 men and 13 women ranging in age from 46 to 79 years (64.7±10.2 years). Adenoma cases having a cancerous component were excluded from this study.

All specimens were fixed in 10% buffered formalin. Carcinomas with adjacent non-neoplastic mucosa were cut serially into 5 mm slices in parallel with the lesser curvature and embedded in paraffin, and then stained with H&E for histological examination.

Immunohistochemistry

Immunohistochemical staining was carried out with antibodies against the following antigens: Cdx2 (BioGenex, San Ramon, CA, USA); MUC5AC (Novocastra Laboratories, Newcastle upon Tyne UK); MUC6 (Novocastra Laboratories); MUC2 (Novocastra Laboratories); and villin (Transduction Labolatories, Lexington, KY, USA); CgA (Dako, Glostrup, Demmark); Gastrin (Yanaihara Institute, Fujinomiya, Japan); Somatostatin (Dako); glucagon-like peptide-1 (GLP-1) (Yanaihara); gastric inhibitory polypeptide (GIP) (Yanaihara); Glicentin (Yanaihara). With regard to gastric phenotypic markers, we used normal gastric mucosa and normal ileum as positive and negative controls, or vice versa, for intestinal phenotypic ones. The precise procedures for immunohistochemical techniques were as previously described (Mizoshita et al., 2003, 2004a,b; Tatematsu et al., 2003; Tsukamoto et al., 2004, 2005; Otsuka et al., 2005). Briefly, 4 μm-thick consecutive sections were deparaffinized and hydrated through a graded series of alcohol. After inhibition of endogenous peroxidase activity by immersion in 3% H_2O_2 /methanol solution, antigen retrieval was conducted by heating in 10 mM citrate buffer (pH 6.0) in a microwave oven for 10 min at 98°C. Sections were incubated with primary antibodies, thoroughly washed in phosphate-buffered saline (PBS), then incubated with biotinylated secondary antibodies, followed by avidin-biotinylated horseradish peroxidase complexes

(Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA, USA). Finally, binding was 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 pathologists (T.T. and T.M.) judged the histology and immunohistochemical staining of the Exo-cell and End-cell markers including Cdx2. The result for CgA staining was evaluated with reference to the percentage of positively stained tumor cells. A result was considered positive if at least 10% of tumor cells were stained. When less than 10% of tumor cells were stained, immunostaining was considered negative.

Classification of tumors

MUC5AC and MUC6 are markers of the gastric Exo-cell phenotype, whereas MUC2 and villin are typical of the intestinal Exo-cell phenotype (Mizoshita et al., 2003, 2004a,b; Tatematsu et al., 2003; Tsukamoto et al., 2005). Similarly, gastrin and somatostatin are markers of the gastric End-cell phenotype, whereas GLP-1, GIP, and glicentin are typical of the intestinal End-cell phenotype (Otsuka et al., 2005).

In the CgA-positive tumor cases, expression of both Exo-cell and End-cell markers was evaluated in tumorous areas having CgA cytoplasmic staining. Firstly, we examined the expression of the End-cell markers in the CgA-positive tumors. Stomach tumorous areas were classified as endocrine-gastric (e-G type) or endocrine-intestinal (e-I type), respectively, with at least one gastric or intestinal End-cell phenotype, and endocrine-gastric-and-intestinal mixed phenotype (e-GI type) when both gastric and intestinal markers were present. Those showing neither gastric nor intestinal phenotypic expression were grouped as endocrine-null type (e-N type). Then, stomach tumorous areas positive for at least one gastric or intestinal Exo-cell marker were classified as of gastric (G type) or intestinal (I type) phenotype, respectively. Those which exhibited both phenotypes were classified as gastric-and-intestinal mixed (GI type), while those showing neither were grouped as null (N type).

Statistical analysis

The data were analyzed by Fisher's exact or χ^2 test for differences between groups. The P-values <0.05 were considered statistically significant.

Results

Expression of CgA in the early and advanced stomach cancers

Totals of 16 (14.5%) and 94 (85.5%) stomach cancers were judged to be CgA-positive and CgA-negative, respectively (Table 1). In the early cases, totals of 4 (10.8%) and 33 (89.2%) lesions were judged to be CgA-positive and CgA-negative, respectively. In the advanced cases, totals of 12 (16.4%) and 61 (83.6%) lesions were judged to be CgA-positive and CgA-negative, respectively. With the histological classification, the CgA-positive rates in cases of the differentiated, and undifferentiated types were 12.5% (7/56) and 16.7% (9/54), respectively, the difference being not significant. There were no significant differences between CgA-positive and CgA-negative groups with reference to age and sex. No lymph node metastasis was observed with the 37 early cases. There was also no significant difference between CgA-positive and CgA-negative groups with reference to lymph node metastasis in the advanced cases. On Kaplan-Meier analysis of the advanced cases, the 5-year survival rates in patients of the CgA-positive and CgA-negative groups were 38.2% and 43.6%, respectively, the difference not being significant (data not shown).

Relations between expression of Exo-cell and End-cell markers in 4 early and 12 advanced CgA-positive stomach cancers

We examined the expression of End-cell markers in 4 early and 12 advanced CgA-positive stomach cancers. Ten (62.5%) cases had the expression of at least one End-cell marker, while 6 cases had no expression of End-cell markers. In 10 CgA-positive cases with End-cell marker expression, we evaluated the expression of the Exo-cell markers (Table 2). In 2 cases (Cases 4 and

Table 1. Correlations between clinicopathological findings and the chromogranin A expression in 37 early and 73 advanced stomach cancers.

Clinicopathological findings	CgA (+) (n=16)	CgA (-) (n=94)	P- value
Age			
Years (mean ± s.d.)	64.0 ± 10.2	60.9 ± 9.7	NS
Sex			
Male (n=67)	12	55	NS
Female (n=43)	4	39	
Histological classification[a]			
Differentiated (n=56)	7	49	NS
Undifferentiated (n54)	9	45	
Depth			
early (n=37)	4	33	NS
advanced (n=73)	12	61	
Lymph node metastasis			
Positive (n=63)	10	53	NS
Negative (n=47)	6	41	

CgA, chromogranin A; NS, not significant; [a]: Classified based on structure of elements. "Differentiated" includes tubular and papillary types, while "Undifferentiated" consists of signet-ring cell and poorly differentiated types.

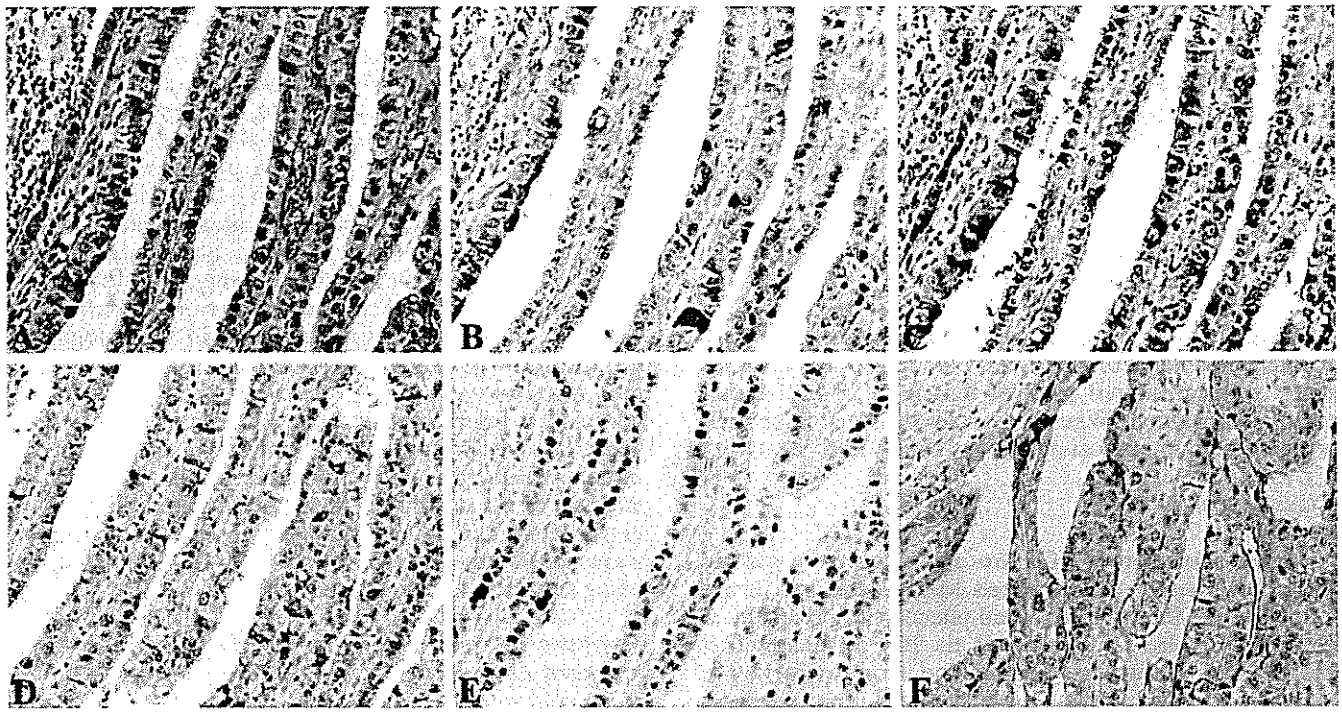

Fig. 1. A stomach cancerous area of e-I type (Case 6f). A. H&E staining. B. CgA cytoplasmic staining observed in some tumor cells. C. GIP is present in the cytoplasm of some cancer cells. D. Glicentin is evident in the cytoplasm of some cancer cells. E. Cdx2 nuclear staining in the tumor cells. F. Villin is positive on the luminal surfaces of cancer cells. CgA, chromogranin A; GIP, gastric inhibitory polypeptide. x 200

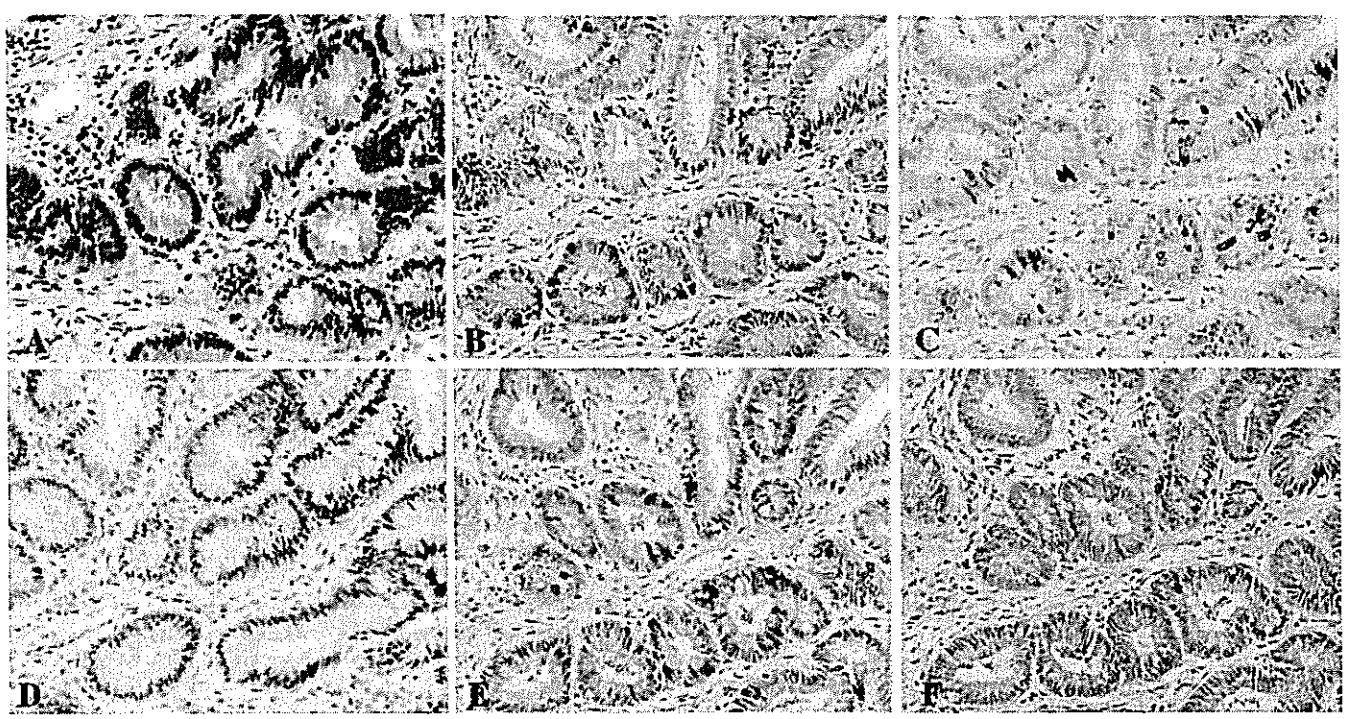

Fig. 2. A stomach adenoma case of e-I type (Adenoma 13). A. H&E staining. B. CgA expression is apparent in the cytoplasm of tumor cells. C. MUC2 is present in the cytoplasm of some adenoma cells. D. Cdx2 is positive in the nuclei of adenoma gland cells. E. GLP-1 is evident in the cytoplasm of some adenoma cells. F. GIP is present in the cytoplasm of some adenoma cells. CgA, chromogranin A; GLP-1, glucagon-like peptide-1; GIP, gastric inhibitory polypeptide. x 200