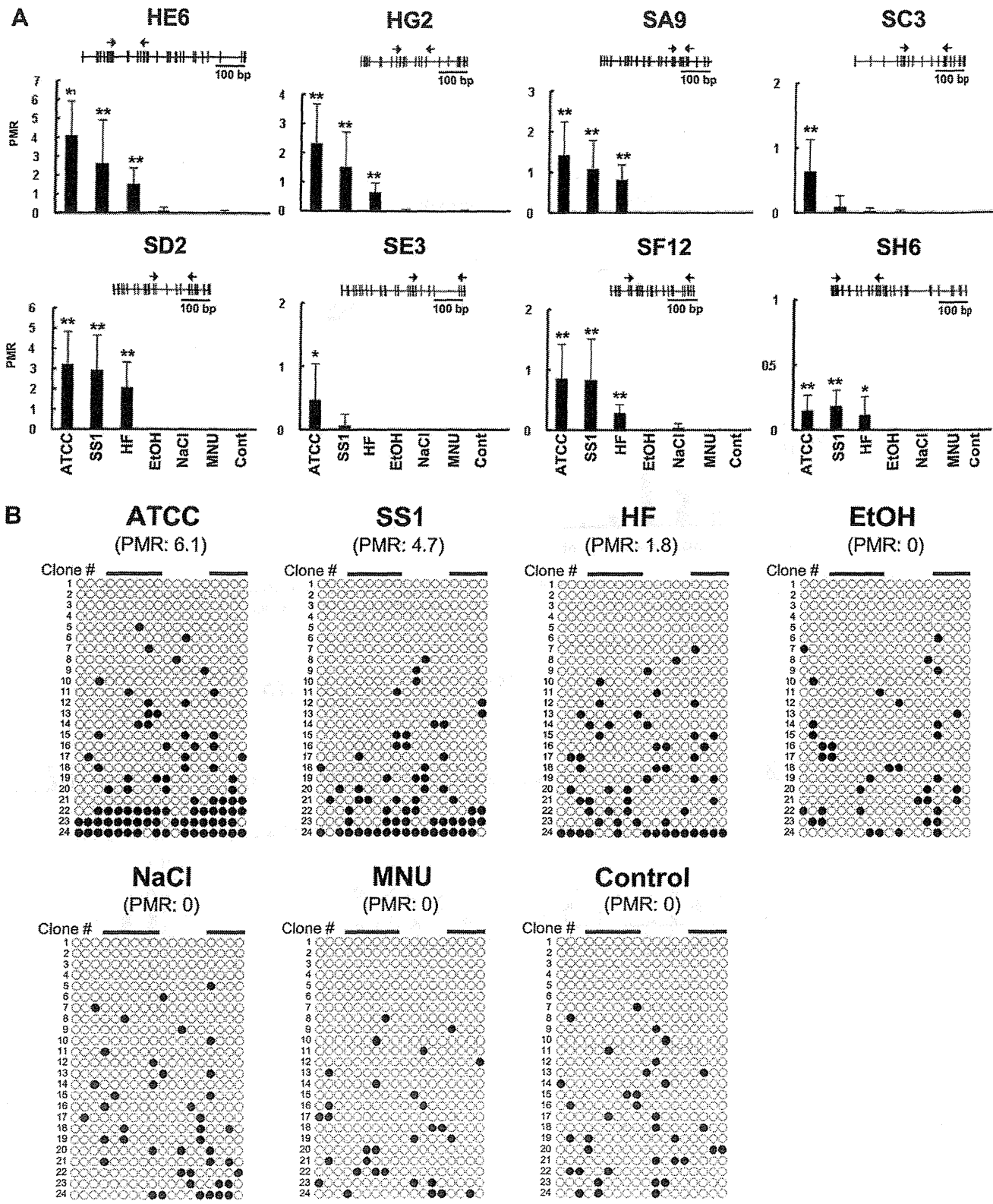

Fig. 2. Methylation induction in GECs by the three *Helicobacter*-induced inflammation but not by EtOH- or NaCl-induced inflammation. (**A**) Methylation levels of eight CGIs assessed by quantitative methylation-specific PCR. Upper panels show CpG maps, and lower panels show methylation levels in percentage of methylated reference. In the upper panel, vertical lines and arrows show individual CpG sites and positions of methylation-specific PCR primers, respectively. Values are shown as mean + SD. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the control group. (**B**) Bisulfite sequencing of HE6 in GECs. Numbers in parentheses indicate percentage of methylated reference of the sample assessed by quantitative methylation-specific PCR. Bars, CpG sites on quantitative methylation-specific PCR primers.

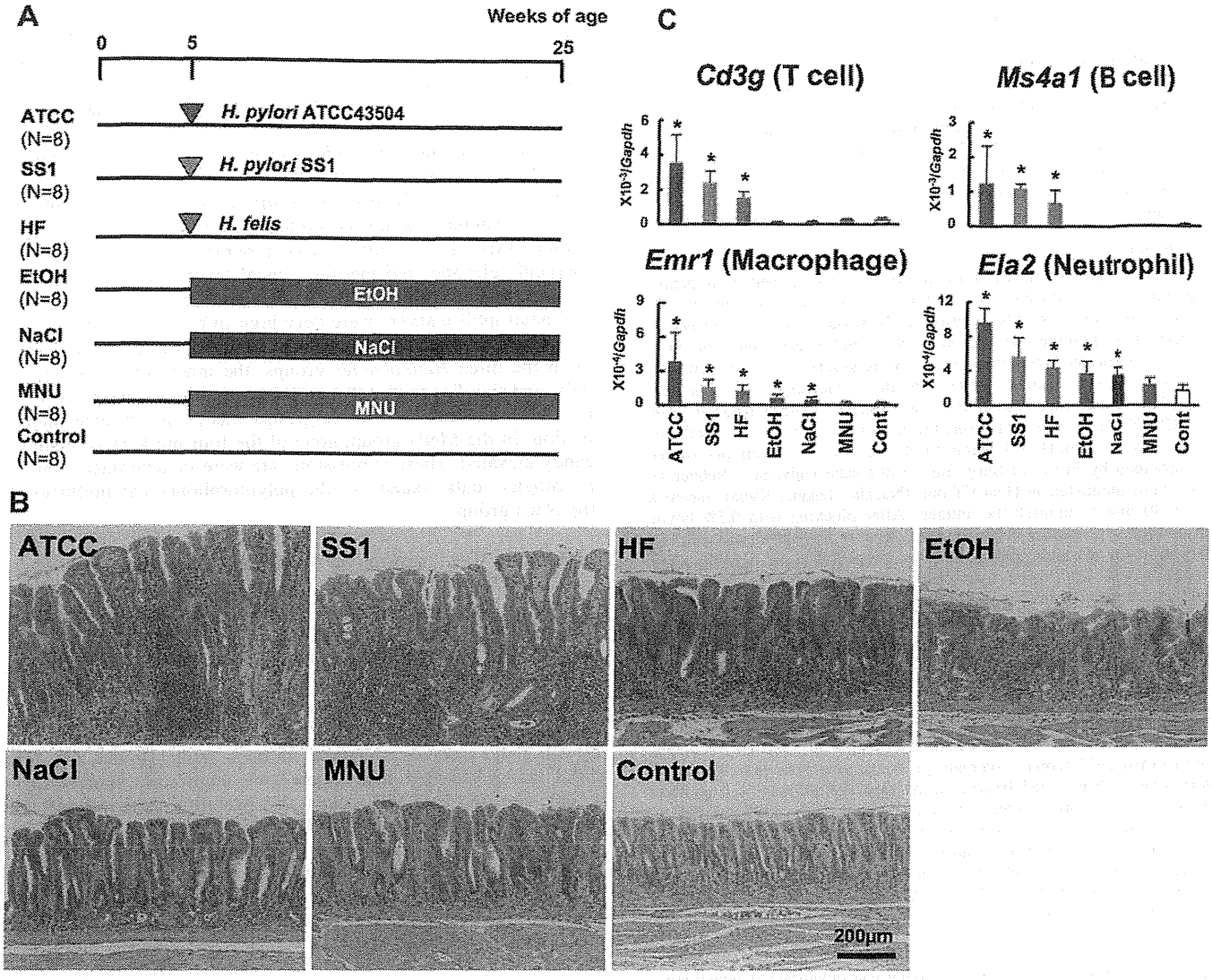

Fig. 1. Treatment of Mongolian gerbils by five inflammation inducers and MNU. (**A**) Experimental design. (**B**) Histology of gastric mucosa after treatment for 20 weeks. Transition of inflammatory cells was observed in the three *Helicobacter* groups. (**C**) Expression levels of inflammatory cell markers. Infiltration of T and B cells was prominent in the three *Helicobacter* groups. Values are shown as mean + SD. $^*P < 0.05$ compared with the control group.

Table I. Histological changes induced by the five inflammation inducers and MNU

Group	Infiltration of mononuclear cells	Infiltration of polymorphonuclear cells	Intestinal metaplasia	Heterotopic proliferative glands
ATCC	2.8 ± 0.5*	2.3 ± 0.7*	0.9 ± 0.6*	1.4 ± 0.9*
SS1	1.6 ± 0.5*	1.1 ± 0.7*	0.0 ± 0.0	0.3 ± 0.5
HF	1.6 ± 0.8*	0.7 ± 0.5*	0.0 ± 0.0	0.4 ± 0.8
EtOH	0.0 ± 0.0	0.9 ± 0.3*	0.0 ± 0.0	0.1 ± 0.3
NaCl	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
MNU	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Control	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

Values are shown as mean ± SD.
$^*P < 0.01$ compared with control group.

SS1, HF, EtOH and NaCl groups but not in the ATCC group. Expression levels of these genes tended to be higher in the EtOH and NaCl groups than in the SS1 and HF groups. The MNU group did not show any significant changes compared with the control group. These results suggested that upregulation of *Il1b*, *Nos2* and *Tnf* was associated with methylation induction.

Expression of Dnmts

Dnmts are the final effectors that methylate DNA (35). To analyze the relation between expression of Dnmts and aberrant methylation induction, we conducted immunohistochemistry of Dnmts. Antibodies against mouse Dnmt1, Dnmt3a and Dnmt3b were tested in gerbils, and those against Dnmt1 and Dnmt3a were confirmed to have high sensitivity and specificity (supplementary Figure 1 is available at *Carcinogenesis* Online).

Dnmt1 protein was localized in the nuclei of GECs around the proliferative zone of gastric glands (supplementary Figures 1 and 2 are available at *Carcinogenesis* Online). In the ATCC, SS1, HF and NaCl groups, the number of GECs expressing Dnmt1 protein was markedly increased and the highest labeling index was observed in the NaCl group (Figure 4B). The profile of Dnmt1 expression was the same as that of Ki-67 (Figure 3B), indicating that Dnmt1 expression was elevated in association with increased cell proliferation. Dnmt3a protein was localized in the nuclei of most GECs except in some cells in the bottom of the glands. Although GECs expressing Dnmt3a protein significantly decreased in the ATCC, EtOH and MNU groups, the degree of decrease was small (Figure 4B and supplementary Figures 1 and 3 are available at *Carcinogenesis* Online). These results showed that the fractions of GECs expressing Dnmt1 and Dnmt3a in gastric glands were not associated with methylation induction.

At age 25 weeks, all the animals were killed, and their stomachs were resected. From the posterior wall of the pyloric region, GECs were isolated by the gland isolation technique (31) for DNA and RNA extraction. The anterior wall of the pyloric region was further cut into two pieces: one for RNA extraction from the mucosal and submucosal layers and the other for histological analysis. DNA and RNA were extracted as described previously (14). As controls in immunohistochemistry of DNA methyltransferases (Dnmts), adult male mice (C57BL/6N, 11 weeks of age; CLEA Japan, Tokyo, Japan) were purchased and stomachs were resected. The animal experiment protocols were approved by the Committee for Ethics in Animal Experimentation.

Histological analysis

After fixation with 10% neutral formalin, tissues were embedded in paraffin and sections at 3 μm thickness were prepared. For histological analysis, hematoxylin and eosin staining was performed by a routine method. The degrees of infiltration of mononuclear and polymorphonuclear cells, intestinal metaplasia and heterotopic proliferative glands were graded on a four-point scale (0–3; 0, no or faint; 1, mild; 2, moderate and 3, marked) as described previously (32). For immunohistochemical analysis, a rabbit anti-human Ki-67 (Clone SP6; Thermo Fisher Scientific, Fremont, CA) antibody was purchased. Rabbit anti-mouse Dnmt1 (33), Dnmt3a (34) and Dnmt3b (34) antibodies were kindly provided by Professor Shoji Tajima at Osaka University. Rehydrated sections were incubated in HistoVT one (Nacalai Tesque, Kyoto, Japan) at 80°C for 40 min to unmask the antigen. After blocking with 0.5% bovine serum albumin in phosphate-buffered saline, sections were incubated with each primary antibody overnight, and the immune complex was visualized by a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). Microscopic images were captured using the BZ-9000 microscope system (Keyence, Osaka, Japan). To analyze the number of the positive cells, more than five gastric glands in at least three different optic fields were counted, and the labeling index was calculated as a percentage of the positive cells relative to the total counted cells.

Human clinical samples

Human gastric mucosae were obtained by endoscopic biopsy from 7 *H.pylori*-negative (4 men and 3 women; average age 70, ranging from 44 to 83) and 18 *H.pylori*-positive (8 men and 10 women; average age 64, ranging from 46 to 81) persons with informed consents and approval of Institutional Review Boards. Their *H.pylori* infection statuses were determined by the serum anti-*H.pylori* IgG test (SBS, Kanazawa, Japan). Endoscopic superficial gastritis was observed in six of the seven *H.pylori*-negative persons and atrophic gastritis was observed in 14 of the 18 *H.pylori*-positive cases. RNA was extracted with ISOGEN (Wako, Osaka, Japan).

Gene expression analysis

The number of complementary DNA molecules was quantified by quantitative reverse transcriptase–polymerase chain reaction (qRT–PCR) as described previously (14). The number of complementary DNA molecules obtained by gene-specific primers (supplementary Table 1 is available at *Carcinogenesis* Online) was normalized to *Gapdh* (*GAPDH*) expression.

Methylation analysis

Methylation levels of gerbil CGIs (HE6, HG2, SA9, SC3, SD2, SE3, SF12 and SH6) were analyzed by quantitative methylation-specific polymerase chain reaction (PCR) and were expressed as a percentage of methylated reference as described previously (14). Bisulfite sequencing was conducted after cloning of PCR products after bisulfite modification as described previously (14).

Statistic analysis

To evaluate significant difference between two independent groups of sample data, the Mann–Whitney *U*-test was employed.

Results

Characterization of five kinds of inflammation triggered by the inducers

Gerbils were treated with five kinds of inflammation inducers (*H.pylori* ATCC 43504, *H.pylori* SS1, *H.felis*, EtOH and saturated NaCl solution) and also with MNU (Figure 1A). By histological examination of the pyloric area, the ATCC group had marked infiltration of mononuclear and polymorphonuclear cells into mucosae and submucosae and glands with intestinal metaplasia and heterotopic proliferative glands were occasionally observed (Figure 1B and Table I). The SS1 and HF groups showed milder infiltration of polymorphonuclear and mononuclear cells, less heterotopic proliferative glands and no intestinal metaplasia. The EtOH group showed infiltration of almost only polymorphonuclear cells. The NaCl group showed no or little infiltration of inflammatory cells but had thickened lamina propria. The MNU group showed no histological inflammatory changes but also had thickened lamina propria.

The kinds of infiltrating inflammatory cells were also assessed by qRT–PCR analysis [*Cd3g* (T cell), *Emr1* (macrophage), *Ela2* (neutrophil) and *Ms4a1* (B cell)] of gastric tissues containing both mucosal and submucosal layers (Figure 1C). In the ATCC, SS1 and HF groups, expression of all the four inflammatory cell markers was markedly elevated and met the typical features of chronic inflammation, such as infiltration of mononuclear cells. The macrophage and neutrophil markers were very high in the ATCC group. In the EtOH and NaCl groups, the neutrophil marker was in the same range as in the three *Helicobacter* groups, the macrophage marker was half, and the T- and B-cell markers were almost absent, showing that the inflammation in these groups was persistent acute inflammation. In the MNU group, none of the four markers were significantly elevated. These expression data were in accordance with the histological data, except for the polymorphonuclear infiltration in the NaCl group.

Induction of DNA methylation by the three Helicobacter strains but not by EtOH and NaCl

To assess methylation in GECs (not in infiltrating leukocytes), we used eight of the 10 CGIs known to be methylated in gerbil GECs as markers because these eight CGIs (HE6, HG2, SA9, SC3, SD2, SE3, SF12 and SH6) have been shown not to be methylated in peripheral blood cells (14). First, methylation levels of these CGIs were measured by quantitative methylation-specific PCR in GECs isolated by the gland isolation technique in each group (Figure 2A). The ATCC group had high methylation levels (significant in all the eight CGIs). The SS1 and HF groups also had high methylation levels (significant in six CGIs; HE6, HG2, SA9, SD2, SF12 and SH6) but lower than the ATCC group. The EtOH, NaCl and MNU groups had no increases of methylation in any CGIs.

To confirm the presence of densely methylated DNA molecules, bisulfite sequencing of HE6 was performed in one gerbil in each group (Figure 2B). Gerbils in the ATCC, SS1 and HF groups had densely methylated DNA molecule(s), and their fractions (3, 1–2, 1 of 24, respectively) were in accordance with the methylation level obtained by quantitative methylation-specific PCR. Gerbils in the EtOH, NaCl and MNU groups had no densely methylated molecules. These data showed that aberrant methylation of these CGIs was induced only by inflammation triggered by the three *Helicobacter* strains, most potently by *H.pylori* ATCC 43504-induced inflammation but not by EtOH- or NaCl-induced inflammation.

Insufficient role of cell proliferation in methylation induction

Cell proliferation was analyzed by immunohistochemistry of Ki-67 in gastric mucosae (Figure 3A) and counting the Ki-67 labeling indices (Figure 3B). All the treatment groups showed significant increases in Ki-67 labeling indices. The three *Helicobacter*-infected groups and the NaCl-treated group showed very high Ki-67 labeling indices. The NaCl-treated group, especially which did not show increased methylation levels, showed the highest Ki-67 labeling index. This result showed that induction of cell proliferation is not sufficient to induce DNA methylation.

Inflammation-related genes associated with methylation induction

To dissect inflammation components responsible for methylation induction, qRT–PCR analysis of 10 inflammation-related genes [*Cox2*, *Cxcl2* (*MIP-2*), *Ifng*, *Il1b*, *Il2*, *Il4*, *Il6*, *Il7*, *Nos2* (*iNos*) and *Tnf* (Tnf-α)] was performed using RNA collected from gastric tissues that contained both GECs and inflammatory cells (Figure 4A). In the three *Helicobacter*-infected groups, *Il1b*, *Nos2* and *Tnf* were significantly upregulated. *Ifng*, *Il2*, *Il4* and *Il6* were significantly upregulated in the

Carcinogenesis vol.32 no.1 pp.35–41, 2011
doi:10.1093/carcin/bgq219
Advance Access publication October 27, 2010

Insufficient role of cell proliferation in aberrant DNA methylation induction and involvement of specific types of inflammation

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Chronic inflammation is deeply involved in induction of aberrant DNA methylation, but it is unclear whether any type of persistent inflammation can induce methylation and how induction of cell proliferation is involved. In this study, Mongolian gerbils were treated with five kinds of inflammation inducers [*Helicobacter pylori* with cytotoxin-associated gene A (CagA), *H.pylori* without CagA, *Helicobacter felis*, 50% ethanol (EtOH) and saturated sodium chloride (NaCl) solution]. Two control groups were treated with a mutagenic carcinogen that induces little inflammation (20 p.p.m. of *N*-methyl-*N*-nitrosourea) and without any treatment. After 20 weeks, chronic inflammation with lymphocyte and macrophage infiltration was prominent in the three *Helicobacter* groups, whereas neutrophil infiltration was mainly observed in the EtOH and NaCl groups. Methylation levels of eight CpG islands significantly increased only in the three *Helicobacter* groups. By Ki-67 staining, cell proliferation was most strongly induced in the NaCl group, demonstrating that induction of cell proliferation is not sufficient for methylation induction. Among the inflammation-related genes, *Il1b*, *Nos2* and *Tnf* showed increased expression specifically in the three *Helicobacter* groups. In human gastric mucosae infected by *H.pylori*, *NOS2* and *TNF* were also increased. These data showed that inflammation due to infection of the three *Helicobacter* strains has a strong potential to induce methylation, regardless of their CagA statuses, and increased cell proliferation was not sufficient for methylation induction. It was suggested that specific types of inflammation characterized by expression of specific inflammation-related genes, along with increased cell proliferation, are necessary for methylation induction.

Introduction

Aberrant DNA methylation of promoter CpG islands (CGIs) is deeply involved in human carcinogenesis (1,2). As inducers of aberrant DNA methylation, aging and chronic inflammation have been suggested because methylation was present in colonic tissues of the aged (3) and patients with long-standing ulcerative colitis (4–6), in the liver with chronic hepatitis (7) and in gastric tissues with *Helicobacter pylori* (*H.pylori*)-induced gastritis (8,9). Especially in the stomach, accumulation levels of aberrant methylation correlate with risk of gastric cancers (8,10–12). Chronic inflammation is characterized by transition of inflammatory cell types from polymorphonuclear cells (mainly neutrophils) to mononuclear cells (lymphocytes and macrophages) and persistent cell proliferation (13). However, it is still unclear whether chronic inflammation with infiltration of mononuclear cells and expression of specific genes or simply persistent inflammation is important for methylation induction and how cell proliferation is involved in it.

As an animal model for methylation induction, we recently demonstrated that inflammation triggered by *H.pylori* infection induces aberrant methylation in the stomach of Mongolian gerbils (*Meriones unguiculatus*) (14). In the gerbil stomach, *H.pylori* with a bacterial virulence factor, cytotoxin-associated gene A (CagA), which is associated with a high risk of human gastric cancers (15), can induce more severe inflammation than that without (16). *Helicobacter felis*, which does not possess CagA (17), can induce chronic gastritis without direct damage of epithelial cells (18,19). High concentrations of ethanol (EtOH) and sodium chloride (NaCl) can induce gastric erosion associated with inflammation (20–22). Their repeated administration can induce persistent inflammation with cell proliferation without transition of inflammatory cell types. In contrast, little inflammation is induced by *N*-methyl-*N*-nitrosourea (MNU), a mutagenic gastric carcinogen (23).

Regarding inflammation-related genes, high expression of *IFNG*, *IL1B*, *TNF*, *NOS2* and *COX2* has been reported in human gastritis induced by *H.pylori* infection (24,25). Also in gerbils, high expression of *Ifng*, *Il1b*, *Cox2* and *Nos2* has been observed (26,27). Our previous time-course study after *H.pylori* infection and eradication in gerbils showed that expression levels of *Cxcl2*, *Il1b*, *Nos2* and *Tnf* were correlated with methylation levels in gastric epithelial cells (GECs) (14). In humans, a polymorphism of *IL1B* is associated with gastric cancer risk (28) and with methylation of multiple genes in gastric cancers (29).

In this study, using five inducers of inflammation (*H.pylori* with CagA, *H.pylori* without CagA, *H.felis*, EtOH and NaCl) and a carcinogen control (MNU), we aimed to clarify the roles of transition of inflammatory cell types, induction of cell proliferation and specific inflammation-related genes in methylation induction.

Materials and methods

Preparation of Helicobacter strains

Helicobacter pylori with CagA (ATCC 43504, also known as NCTC 11637) was obtained from the American Type Culture Collection (ATCC, Rockville, MD). *Helicobacter pylori* without CagA, SS1, was kindly provided by Professor Takashi Joh at Nagoya City University (30). *Helicobacter felis* (ATCC 49179) was also obtained from ATCC. Each strain was inoculated in Brucella broth (Becton Dickson, Cockeysville, MD) with 7% vol/vol heat-inactivated fetal bovine serum and incubated at 37°C under microaerobic conditions using an AnaeroPack Campylo (Mitsubishi Gas Chemical, Tokyo, Japan) for 24 h. For the culture of *H.felis*, 0.1% wt/vol of BactoAgar (Becton Dickson) was supplemented. Before harvesting bacteria, their mobility and shape were confirmed under phase contrast microscopy.

Animal experiments and sample preparation

Five-week-old male Mongolian gerbils (MGS/Sea; Kyudo, Tosu, Japan) were randomly assigned to seven groups of eight animals each. Gerbils in groups for *Helicobacter* treatment were inoculated with ~10^8 CFU/gerbil of *H.pylori* ATCC 43504 (ATCC group), *H.pylori* SS1 (SS1 group) or *H.felis* (HF group) and were kept without further treatment. Gerbils in groups of EtOH and NaCl treatment were administered with 5 ml/kg body wt of 50% EtOH group and saturated NaCl group, respectively, by gavage twice a week from 5 to 25 weeks of age. Gerbils in the group of MNU treatment (MNU group) were administered with 20 p.p.m. of MNU (Sigma–Aldrich, St Louis, MO) in drinking water from 5 to 25 weeks of age. A control group was kept without any treatment.

Abbreviations: CagA, cytotoxin-associated gene A; CGI, CpG island; Dnmt, DNA methyltransferase; EtOH, ethanol; GEC, gastric epithelial cell; MNU, *N*-methyl-*N*-nitrosourea; NaCl, sodium chloride; PCR, polymerase chain reaction; qRT–PCR, quantitative reverse transcriptase–polymerase chain reaction.

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>6.0% for the control group. This high incidence rate is probably due to the fact that, in those previous studies, the extent of CAG together with intestinal metaplasia in background stomachs of study patients was not evaluated. Subjects were thus probably heterogeneous in the degree of CAG, which was probably milder as a whole compared to that in subjects in the present study. The observed high incidence rate of metachronous cancer development is evidence for a strategy for cancer prevention in these subjects other than regular and strict follow-up by endoscopy. As described above, subjects with metaplastic gastritis, an *H. pylori*-negative lesion, cannot be treated with *H. pylori* eradication. Furthermore, high expression levels of COX-2 in intestinal metaplasia have been observed even after *H. pylori* eradication.[47] Treatment with a selective COX-2 inhibitor thus appears to represent a reasonable option for subjects with metaplastic gastritis, rather than regular follow-up for cancer.

Although the present study revealed preventive effects of a selective COX-inhibitor, etodolac, on metachronous cancer development in curatively treated gastric cancer patients with metaplastic gastritis, the study shows some limitations. First, the present study was prospectively conducted, but treatment with etodolac was not randomized. However, randomization was not feasible, as most eligible, high-risk cancer subjects were unwilling to remain untreated for long periods, particularly with the knowledge of the results of previous epidemiologic studies that long-term use of NSAIDs, including aspirin, is associated with a reduced risk of gastric cancer.[35,36] In addition, the number of subjects was small because the incidence of serologically diagnosed metaplastic gastritis is quite low, comprising <1% of the middle-aged Japanese population[31,32] and <20% of total gastric cancer cases. Considering the fact that *H. pylori* eradication does not completely eradicate cancer[7–12] and that eradication might be effective in the control of cancer development only among subjects with mild CAG,[7,10] posteradication subjects with extensive CAG and intestinal metaplasia should be considered another possible target for treatment with selective COX-2 inhibitors. However, special attention should be paid to recent evidence that long-term use of COX-2 inhibitors is associated with increased cardiovascular risk, including not only thrombotic events, but also hypertension, congestive heart failure, and arrhythmic events.[48–50] Since the reported cardiovascular toxicity of COX-2 inhibitors is variable among the different drugs and with the dose of each particular drug and, based on past data, etodolac treatment at a dose of 300 mg/day appears to be relatively low in cardiotoxicity,[51,52] the present results warrant a prospective randomized trial. Further careful study using the present dose of etodolac and avoiding inclusion of patients with increased risk of cardiovascular complications would contribute greatly to determining the effectiveness and safety of long-term chemopreventive treatment.

In conclusion, the present results strongly indicate that selective COX-2 inhibitors provide a potent strategy for tertiary cancer prevention in curatively treated gastric cancer patients with metaplastic gastritis, an *H. pylori*-negative premalignant lesion.

Acknowledgements

The authors would like to express their deepest thanks to Ms. Kazu Konishi for her excellent secretarial assistance.

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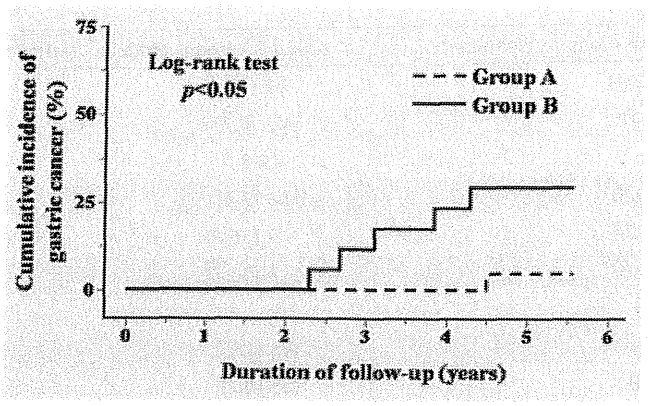

Figure 1. Kaplan–Meier analysis of metachronous cancer development in patients with early gastric cancer resected endoscopically. Group A received etodolac treatment (300 mg/day), while Group B did not receive any treatment. Both groups were followed for up to 5 years. Cancer incidence rates were 898/100,000 person-years for Group A and 6,266/100,000 person-years for Group B, showing a significant difference in cancer development rates between groups ($p = 0.05$; log-rank test).

no significant differences between groups. All cancers in these 47 patients were resected by ESD.

Patients were followed for up to 5 years. Mean (standard deviation) follow-up period was 4.2 (0.9) years. By the end of the study period, cancer development was observed in 1 Group A patient and 5 Group B patients. As shown by Kaplan–Meier analysis, cancer development in the Group A patient was observed 5 years after the start of the study. In contrast, cancer development occurred steadily throughout the study period in Group B patients (Fig. 1). Cancer incidence rates in Groups A and B were 898/100,000 person-years and 6,266/100,000 person-years, respectively, representing a significant difference ($p < 0.05$, log-rank test). Cancers that developed in these patients were all intestinal-type mucosal cancers on histopathology, and sizes were <10 mm in diameter. All these lesions were thus resected endoscopically.

The extent of CAG together with intestinal metaplasia as revealed by endoscopic findings did not change significantly in Group B patients during the study period. In addition, the difference between serum PG levels for each patient at the start compared to the end of the study period was not significantly different and was within the range of interassay variation. Etodolac treatment did not exert any influence on the extent of metaplastic gastritis in Group A patients and did not induce any other specific change in endoscopic findings except for a single case of gastric erosion observed in the prepyloric antrum of 1 patient. Serum PG levels of Group A patients were also unaltered by etodolac treatment. This medication was well tolerated by all patients during the study period.

Discussion

In the present study, long-term treatment with a selective COX-2 inhibitor, etodolac, effectively inhibited metachronous cancer development in curatively treated, early gastric cancer patients with metaplastic gastritis. These results are in line with the results of our previous animal experiment using *H. pylori*-infected Mongolian gerbils,[19] indicating that etodolac can prevent stomach carcinogenesis involving the CAG-metaplasia-dysplasia-cancer sequence. Essentially, the same results have also been reported with the use of another selective COX-2 inhibitor, celecoxib.[20] Furthermore, previous epidemiologic studies have demonstrated that long-term nonselective inhibition of COXs (COX-1 and COX-2) by NSAID treatment is effective for preventing gastric cancer.[35,36] However, evaluation of the preventive effects of selective COX-2 inhibition on gastric cancer by 2 epidemiologic studies investigating the regression of intestinal metaplasia as a primary parameter (a surrogate parameter for cancer prevention) revealed conflicting results. One randomized controlled study indicated that rofecoxib treatment had no significant effect on the regression of intestinal metaplasia,[37] whereas the other nonrandomized study indicated a beneficial effect of celecoxib.[38] The contradictory outcomes of these studies could be partially explained by the differential effects of selective COX-2 inhibitors according to dose, type of drug and duration of exposure,[39] but also, and more importantly, by differences in severity of the target lesion—the extent of coexisting CAG together with intestinal metaplasia—among study patients. The present results indicate that the extent of premalignant lesions as revealed by serum PG levels did not change significantly during the study period despite etodolac treatment. Since the study period was not long, further long-term investigations are warranted to determine the inhibition of progression and/or regression of metaplastic gastritis by COX-2 inhibition. Nonetheless, our results strongly indicate that COX-2 is deeply involved in the growth of initiated cells in the metaplastic stomach and that etodolac treatment leads to a marked delay in cancer development.

We selected early gastric cancer patients with metaplastic gastritis as a target for treatment with a selective COX-2 inhibitor. Several previous studies have demonstrated that the more advanced the stage of *H. pylori*-related CAG, the greater the cancer risk.[31,32,40–44] Subjects with metaplastic gastritis, an end result of long-lasting *H. pylori* infection, are thus considered to be at particularly high risk of gastric cancer. Indeed, our previous longitudinal cohort study found that a group of middle-aged male subjects with metaplastic gastritis based on 2 serum tests—negative results for *H. pylori* antibody and positive results on the PG test—displayed an annual cancer incidence rate of about 0.87%, meaning that 1 cancer developed in 11.5 subjects during every 10-year period.[31,32] Subjects selected for the present study were early cancer patients curatively treated with endoscopic resection and thus appear to constitute a subgroup at even higher risk for gastric cancer among subjects with metaplastic gastritis. A few previous studies have reported an annual incidence of metachronous cancer after endoscopic resection of about 1.3–4.0%,[45,46] while the annual cancer incidence rate in the present study was

Table 1. Profiles of subjects in groups A and B

	Group A with etodolac treatment	Group B without etodolac treatment
Number of subjects (male:female)	26 (22:4)	19 (17:2)
Follow-up, years [mean (SD)]	4.3 (1.1)	4.2 (0.7)
Person-years	111.4	79.8
Age, years [mean (SD)]	71.3 (10.2)	70.6 (7.4)
Alcohol drinking, *n* (%)	10 (38.4)	7 (36.8)
Smoker, *n* (%)	9 (34.6)	7 (36.8)
Serum PG levels at the start of the study		
PGI, mg/ml [mean (SD)]	21.4 (18.4)	20.1 (18.2)
PG I/II [mean (SD)]	1.7 (0.8)	1.5 (0.7)
Serum PG levels at the end of the study		
PGI, mg/ml [mean (SD)]	18.2 (14.1)	18.0 (12.4)
PG I/II [mean (SD)]	1.5 (0.8)	1.6 (0.7)
Total gastric cancer developed		
Case/incidence rate[1]	1/898	5/6266[2]
Details of the resected cancers		
Size, mm [mean (SD)]	31.5 (13.6)	32.4 (17.4)
Location [upper/middle/lower (%)][3]	14/10/2 (54/38/8)	11/7/1 (58/37/5)
Macroscopic type [IIa/IIb/IIc (%)]	18/3/5 (69/12/19)	12/2/5 (63/11/26)
Depth of invasion, *n* of mucosal cancer (%)	26 (100)	19 (100)
Histopathology type, *n* of intestinal type (%)	26 (100)	19 (100)
Synchronous multiple cancer cases, *n* (%)	2 (8)	1 (5)
Method of endoscopic resection, *n* of ESD (%)	26 (100)	19 (100)

[1]Per 100,000 person-years. [2]$p < 0.05$ (*vs.* Group A with etodolac treatment). [3]Location and macroscopic type of the cancer were determined according to the Japanese Classification of Gastric Carcinoma (Ref. 34).

of each review. Written informed consent was obtained from all participating patients. The Committee on Ethics at Wakayama Medical University approved all study protocols.

Evaluation of cancer histopathology

Resected specimens of gastric cancer obtained by endoscopy were assessed histopathologically and classified according to Lauren's classification into intestinal or diffuse type.[33] Location and macroscopic type of the cancer in the stomach were classified based on clinical and histopathological records according to the classifications of the Japanese Gastric Cancer Association.[34]

Statistical analysis

Data were analyzed using SPSS 11.0 software (SPSS, Chicago, IL) and STATA software (STATA, College Station, TX). Differences were tested for significance using the Mann–Whitney *U*-test for comparisons between 2 groups. The chi-square test and Fisher's exact test were used to compare categorical variables. Long-term effects of etodolac on gastric cancer development were analyzed by the Kaplan–Meier method, and statistical differences between curves were tested by the log-rank test. For all comparisons, p values less than 5% ($p < 0.05$) were considered statistically significant.

Results

Among the 47 patients with endoscopically and serologically diagnosed metaplastic gastritis who underwent endoscopic resection for early gastric cancer, 26 received etodolac treatment (Group A) and the remaining 21 did not receive any treatment (Group B). These 2 groups of patients were followed and development of gastric cancer was investigated. During the first year of the study, 2 patients in Group B developed cancer. One cancer case was detected 8 weeks after resection and the other 6 months later. In both cases, the cancerous lesions were able to be retrospectively identified on endoscopic images from before resection. These cancers were considered to be synchronous cancers and were thus excluded from the study, and the remaining 45 patients were analyzed. Table 1 shows baseline characteristics for the 2 groups. No significant differences in age, sex distribution, or lifestyle factors at baseline were apparent between groups. In addition, the extent of CAG together with intestinal metaplasia at the time of mucosal resection as evaluated by endoscopic findings was similar between groups, as were serum PG levels. Furthermore, comparison of clinicopathological features (size, location, macroscopic type, depth of invasion, histopathological type, *etc.*) of the resected cancers revealed

Anticancer effects of 4-vinyl-2,6-dimethoxyphenol (canolol) against SGC-7901 human gastric carcinoma cells

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Received December 12, 2012; Accepted February 27, 2013

DOI: 10.3892/ol.2013.1230

Abstract. Gastric cancer remains the fourth most commonly diagnosed cancer and is the second leading cause of cancer-related mortality worldwide. The aim of this study was to investigate the effects of canolol on the proliferation and apoptosis of SGC-7901 human gastric cancer cells and its relevant molecular mechanisms. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to observe the effect of canolol on the proliferation of SGC-7901 human gastric adenocarcinoma cells. The results showed that SGC-7901 cells exhibited a marked dose-dependent reduction in the proliferation rate. The survival rate of the cells was 88.86±1.58% at 50 μmol/l, decreasing to 53.73±1.51% at 800 μmol/l ($P<0.05$). By contrast, canolol had no significant toxicity on the human gastric mucosal epithelial cell line GES-1. The vivid images of cell morphology using an inverted microscope provided confirmation of the MTT assay. Treatment of SGC-7901 cells with canolol resulted in apoptosis demonstrated by flow cytometry. Furthermore, canolol downregulated the mRNA levels of COX-2, but had no significant effect on the mRNA expession of the Bax and Bcl-2 genes. These findings suggest that canolol has potential to be developed as a new natural anti-gastric carcinoma agent.

Introduction

Gastric cancer remains the fourth most commonly diagnosed cancer and is the second leading cause of cancer related mortality worldwide (1). Gastric cancer is the most common cancer in Eastern Asia (2). Eradication of *H. pylori* in the stomach by administration of oral antimicrobial agents results in the resolution of *H. pylori*-infected chronic active gastritis and significantly reduces the risk of gastric cancer development (3). However, bacterial eradication treatment has been lacking. The occurrence of antibiotic-resistant *H. pylori* has been reported (4) and is occasionally associated with adverse effects. Regular therapies such as chemotherapy, biotherapy and radiotherapy have been previously applied, however, they have unavoidable side effects (5). Therefore, more effective alternative approaches for gastric cancer prevention and therapies without undesirable side-effects are needed.

It is widely accepted that phytochemical, especially phenolic, compounds are associated with anticancer effects by affecting molecular events in the initiation, promotion and progression stages. Recent studies have demonstrated protective effects of plant phenolic compounds against gastric cancer (6-8). The expansion ability of tumor cells depends on the rate of both cell proliferation and cell apoptosis. The particular features of tumor cells allow them to evade apoptosis, a cell suicide program that reduce the damaged or mutated cells to maintain homeostasis (9).

Canolol, 4-vinyl-2,6-dimethoxyphenol (Fig. 1), is purified from crude canola oil and is a novel and potent antioxidant. Canolol has been proven to prevent *H. pylori*-induced gastritis and carcinogenesis in an animal model (10). However, its potential anti-proliferative and proapoptotic effects on gastric cancer cells and the possible mechanisms remain unknown.

The role of cyclooxygenase-2 (COX-2) inhibitors in the chemoprophylaxis of gastric cancer has been investigated. COX-2, the inducible isoform of COX, is undetectable in normal tissues and highly expressed in gastric tumors (11). Experimental studies have identified the correlation between COX-2 overexpression and the increased cell proliferation and decreased cell apoptosis in malignant tumor cells (12,13). COX inhibitors (Coxibs) are a series of drugs with analgesic, antipyretic and anti-inflammatory properties. Evidence suggests that COX-2 inhibitors correlate with tumor inhibition in breast (14) and endometrial cancer cell lines (15). Induction of apoptosis has increasingly become important with regard to the mechanism of cancer defense and prevention (16). However, the involvement of COX-2 inhibitors in gastric cancer prophy-

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Key words: canolol, gastric cancer, COX-2, anti-proliferation, apoptosis

laxis remains to be determined, as the long-term use of COX-2 inhibitors exerts side-effects on the cardiovascular system and the digestive tract. A possible correlation between COX-2 inhibition and cell apoptosis in gastric cancer cell lines has yet to be examined.

In the present study, the effects of canolol on growth and apoptosis of human gastric adenocarcinoma SGC-7901 cells were investigated. Human gastric mucosal epithelial (GES-1) cells were used as the control cell model to examine the non-specific cytotoxicity of canolol. The mRNA expression levels of COX-2, Bcl-2 and Bax were detected to further elucidate the possible mechanisms involved.

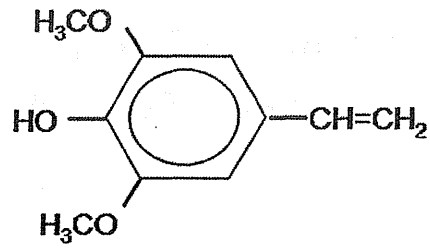

Figure 1. Chemical structure of canolol. 4-vinyl-2,6-dimethoxyphenol. Molecular weight: 180.

Materials and methods

Materials and reagents. 4-Vinyl-2,6-dimethoxyphenol (canolol with a molecular mass of 180) was purchased from Junsei Chemical, Tokyo, Japan. It was synthesized to at least 95% purity (confirmed by nuclear magnetic resonance). The preparation was sealed under helium or nitrogen and maintained at -80˚C. Canolol was dissolved in ethanol and diluted in a serum-free medium immediately before the experiments. Gastric cancer SGC-7901 cells were obtained from the Department of Pathogeny Biology, Norman Bethune Medical College of Jilin University, China. Human gastric mucosal epithelial cell line GES-1 was obtained from the Cancer Hospital of Beijing University. The study protocol was approved by the ethics committee of the First Hospital of Jilin University.

Cell culture and treatment. Human SGC-7901 gastric cancer cell line and human GES-1 gastric mucosal epithelial cell line were cultured in RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum (FBS) and 100 ng/ml each of penicillin and streptomycin in an incubator (50 ml/l CO_2) at 37˚C. The medium was changed every 2-3 days. Cells in the logarithmic growth phase were collected for subsequent experiments. The cells were treated with various concentrations of canolol for 24 h.

Cell viability assay. The method of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was employed to determine cell viability. Cultured SGC-7901 and GES-1 cells were detached using trypsinization, centrifuged at 1,000 x g for 5 min and resuspended in fresh RPMI-1640 medium. The cells were plated at a density of $5x10^3$ cells/well in 96-well microplates and treated with canolol ranging from 25 to 1,200 μmol l for 24 h at 37˚C. At the end of treatment, 20 μl of MTT stock solution was added to each well [(0.5 mg/ml in phosphate-buffered saline (PBS)] for 4 h. The medium was replaced with 150 μl DMSO to dissolve the converted purple dye in the culture plates. Absorbance was measured at 570 nm on a spectrophotometer microplate reader. Cell viability was assayed as the relative formazan formation in treated compared with control wells after correction for background absorbance. Four wells per dose were counted in each experiment. Analyses were performed using SPSS version 10.0 (SPSS Inc, Chicago, IL, USA). Data were evaluated using one-way ANOVA. $P<0.05$ was considered statistically significant.

Cell morphology. SGC-7901 and GES-1 cells were seeded at a density of $5x10^5$ cells/well onto a cover slip loaded in 6-well plates. Fresh RPMI-1640 medium containing different concentrations of canolol was added. Cells were photographed with an inverted microscope under x200 magnifications to observe morphological changes.

Annexin V-FITC/PI staining for flow cytometry. SGC-7901 cells were collected and centrifuged at 1,000 x g for 5 min and resuspended in fresh RPMI-1640 medium at a density of $2x10^5$ cells/ml. Apoptotic and necrotic cells were evaluated by Annexin V (AV) binding and propidium iodide (PI) uptake using an AV-FITC-PI apoptosis assay kit (Pharmingen, San Diego, CA, USA). Samples were analyzed by flow cytometry.

Real-time quantitive PCR analysis. Total RNA of SGC-7901 cells was extracted using an RNA extraction kit and primers used are shown in Table I. Following DNase treatment, the first strand cDNA was synthesized. Quantitive PCR of Bcl-2, Bax and COX-2 were performed with the Bio-Rad (Hercules, CA, USA) CFX system. To exclude variations caused by RNA quantity and quality, the GAPDH gene was used as an internal control. Analyses were performed using SPSS version 10.0 (SPSS Inc). Data were evaluated using one-way ANOVA. $P<0.05$ was considered a statistically significant result.

Results

Canolol does not exhibit evident toxicity to GES-1 cells. The proliferation effect of canolol was determined using an MTT assay and GES-1 cells were used as a control to detect the cell toxicity of canolol. Cells were treated with different concentrations of canolol (0-1200 μmol l). The data indicated that canolol has no obvious cytotoxicity against normal GES-1 cells. The percentage of cell viability was 99.38±3.57% at 25 μmol l, 87.82±2.55% at 800 μmol l and decreased to 65.31±4.44% at 1200 μmol l (Fig. 2). Cell morphology using an inverted microscope also showed that cell structures were intact and were well established after 1,200 μmol l canolol treatment (Fig. 3).

Canolol inhibits proliferation and induces apoptosis of SGC-7901 cells. SGC-7901 cells were treated with different concentrations of canolol (0-1200 μmol l). The percentages of cell viability at various canolol doses were determined as the percentage of viable treated cells in comparison with

Table I. Primer sequences used in real-time quantitive PCR.

Gene	Primer sequence	Annealing temperature (°C)	Product size (bp)
COX-2	F: CTCCCTTGGGTGTCAAAGGTA R: GCCCTCGCTTATGATCTGTC	76	171
Bcl-2	F: GAGTTCGGTGGGGTCATG R: GGAGAAATCAAACAGAGGC	83	186
Bax	F: GGATGCGTCCACCAAGAA R: GAGCACTCCCGCCACAAA	83.5	388
GAPDH	F: AACGGATTTGGTCGTATTG R: GGAAGATGGTGATGGGATT	78.5	258

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; F, forward; R, reverse.

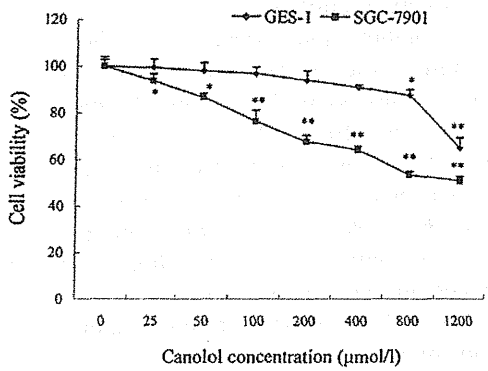

Figure 2. Effect of canolol on cell viability under different concentrations on particular cells using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (mean ± SD) (n = 4). Data were evaluated using one-way ANOVA. *P<0.05, **P<0.01.

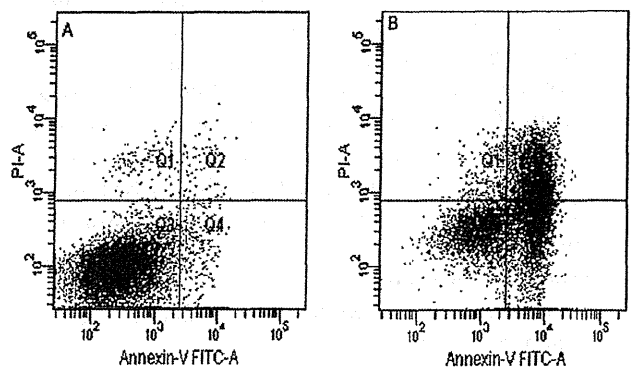

Figure 4. Apoptosis of SGC-7901 cells was investigated using a flow cytometry assay using FITC-Annexin-V/PI staining. (A) SGC-7901 cells without canolol; (B) SGC-7901 cells with 400 μmol/l canolol.

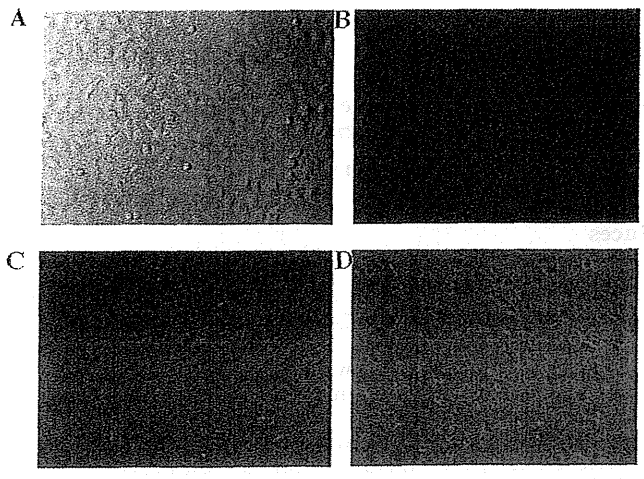

Figure 3. Morphology of GES-1 and SGC-7901 cells treated with 1,200 μmol l canolol.

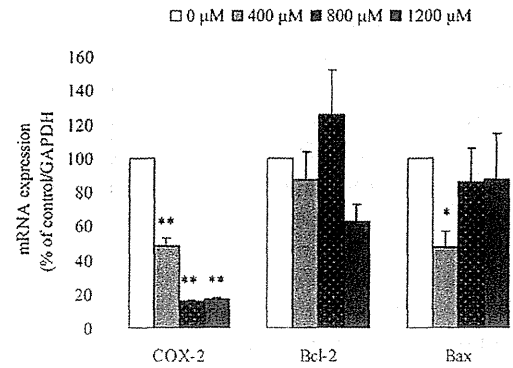

Figure 5. Relative expression levels of COX2, Bcl-2 and Bax mRNAs in SGC-7901 cells under treatment of different concentrations of canolol (mean ± SD) (n=3). Values are arbitrary unit values (mean ± SD) relative to 100 for controls. GAPDH was used as an internal control. Data were evaluated using one-way ANOVA. *P<0.05, **P<0.01 vs. control

viable untreated cells. The results provided solid evidence that the inhibitory effects on the proliferation of canolol to SGC-7901 cells were dose-dependent (Fig. 2): the percentage of cell viability was 89.80±2.83% at 25 μmol l, 73.73±1.51% at 800 μmol l (P<0.05) and 51.22±1.82% at 1,200 μmol l (P<0.01). Consistent with the MTT assay results, the adherent SGC-7901 cells were markedly decreased and showed apoptosis under the treatment of 1,200 μmol l canolol (Fig. 3).

Furthermore, a flow cytometric analysis was used to quantify the rate of cell apoptosis using double staining of Annexin

V-FITC and PI. As shown in Fig. 4, the lower right field (high Annexin V, low PI staining) represents the early apoptotic cells due to the strong affinity of Annexin V-FITC with phosphatidylserine, which transports from the inner to the outer surface of the plasma membrane during early apoptosis. By contrast, the higher left field (high PI, low Annexin V staining) represents the necrotic cells, since PI, which binds to nucleic acids, only cross through the compromised membrane of dead cells or late apoptotic cells (17). Viable cells are shown in the lower left field (low Annexin V and PI staining) and the higher right field (high Annexin V and PI staining), indicating late apoptotic cells. The results showed that canolol was able to induce the apoptosis of SGC-7901 cells and the rate of early apoptosis, late apoptosis and necrosis of SGC-7901 cells were increased under 400 μmol/l canolol (Fig. 4).

Canolol downregulates the mRNA expression level of COX-2. To clarify the mechanisms of SGC-7901 cell apoptosis under canolol treatment, the mRNA expression level of COX-2, Bcl-2 and Bax was evaluated using real-time quantitive PCR. The sequences of these primers were shown in Table I. The results showed that in SGC-7901 cells, the relative mRNA expression level of COX-2 was decreased to 48.50±4.67% in 400 μmol/l, 16.08±0.75% in 800 μmol/l and 17.22±0.88% in 1,200 μmol/l canolol. The effect of canolol on COX-2 expression was downregulated ($P<0.01$); However, the expression levels of Bcl-2 and Bax fluctuated slightly (Fig. 5). These data suggested that the inhibition of COX-2 might play an important role in the apoptosis of SGC-7901 cells.

Discussion

Gastric cancer is one of the most prevalent malignant tumors and its morbidity is the highest in China. Currently, many natural and synthesized compounds are used in the chemoprovention and treatment of gastric cancer (18,19). Canolol, 4-vinyl-2,6-dimethoxyphenol, which is extracted from crude canola oil, has the ability to prevent *H. pylori*-infected gastric carcinogenesis in gerbils (10). In the present study, it was demonstrated that canolol prevented proliferation and induced apoptosis of SGC-7901 cells dose-dependently *in vitro*. Additionally, it had low toxicity to immortalized GES-1 cells (Figs. 2 and 3). The results indicated that canolol has the potential to be developed as a new natural anti-gastric carcinoma agent.

COX-2 is important in the conversion of arachidonic acid to prostaglandin H_2. Accumulating evidence suggests that the constitutive overexpresion of the inducible COX-2 gene is involved in a diverse array of cancers and Harris *et al* (20) demonstrated that COX-2 overexpresion initiated and promoted carcinogenesis through: i) mutagenesis, i.e., the production of certain reactive oxygen species that are carcinogenic; ii) mitogenesis, i.e., cell proliferation promoted by PGE-2 and other factors; iii) anti-apoptosis, i.e., cell differentiation and apoptosis reduced by PGE-2 and other factors; iv) angiogenesis, metastasis and immunosuppression (20). The real-time quantitive PCR in this study showed that COX-2 expression was downregulated under canolol treatment ($P<0.01$) (Fig. 5). It was postulated that inhibition of COX-2 expression may result in blockade of the prostaglandin cascade and a decrease in reactive oxygen species (ROS), thus stimulating apoptosis of malignant cells and preventing neoplastic growth. The scavenging potency of canolol against $ROO^{\cdot}$ is much higher than that of well-known antioxidants, such as α-tocopherol, vitamin C and β-carotene (21). A previous study in this laboratory showed canolol decreased serum 8-OHdG, a key biomarker of oxidative DNA damage relevant to carcinogenesis (10). Other natural phenolic extracts, such as BCE (black currant extract) and dioscin, reduce the risk of gastric cancer owing to their antioxidative functions (22-24).

Selective and non-selective COX-2 inhibitors may be involved in the intervention and chemoprevention of carcinogenesis (25-27). A series of epidemiologic studies found that the COX-2 inhibition levels of coxibs were consistent with their chemopreventive effects in cancers of the breast, colon, prostate and lung (20). Ma *et al* (28) have demonstrated that PGE2 acts with a family of G-protein-coupled receptors participating in multiple signal tranduction pathways.

The Bcl-2 family, such as Bax, Bad, Bid, Bcl-2 and Bcl-x, is one of the most extensively studied groups of proteins involved in cell apoptosis. Bax, Bad and Bid were shown to activate apoptosis, while Bcl-2 and Bcl-x were shown to inhibit the process (29). Transfection of COX-2 constitutive expression vector into the BCC cell line significantly upregulated Bcl-2 expression and this indicated that Bcl-2 might participate in COX-2 mediated anti-apoptic processes (30). In addition, the expression level of Bax, a member of a pro-apopotic protein family was downregulated in a transgenic mouse model (31). However, in the present study, no correlation between Bcl-2/Bax and COX-2 expression was found (Fig. 5).

The relationship between apoptosis and COX-2 downregulation in this gastric adenocarcinoma cells should be studied. COX-2 is a potential pharmacologic target that may be used in the prevention and treatment of various types of malignancies.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (Nos. 30972476 and 81273065). The authors would like to thank Dr Yu Chen for technical support.

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Semin Immunopathol (2013) 35:177–190
DOI 10.1007/s00281-012-0357-1

REVIEW

Helicobacter pylori infection and gastric carcinogenesis in rodent models

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Received: 24 July 2012 / Accepted: 15 October 2012 / Published online: 31 October 2012

Abstract *Helicobacter pylori* infection is an important factor for gastric carcinogenesis in human. In carcinogen-treated Mongolian gerbils, *H. pylori* infection enhances stomach carcinogenesis, while infection alone induced severe hyperplasia called heterotopic proliferative glands. A high-salt diet or early acquisition of the bacteria exacerbates inflammation and carcinogenesis. Oxygen radical scavengers or anti-inflammatory chemicals as well as eradication of *H. pylori* are effective to prevent carcinogenesis. *H. pylori*-associated inflammation induces intestinal metaplasia and intestinalization of stomach cancers independently. It is necessary to control cancer development not only in *H. pylori*-positive cases but also in *H. pylori*-negative metaplastic gastritis.

Keywords *Helicobacter pylori* · Mongolian gerbil · Intestinal metaplasia · Gastric adenocarcinoma · Chemoprevention

This article is a contribution to the special issue on Inflammation and Cancer - Guest Editor: Takuji Tanaka

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Introduction

Gastric cancer is in decreasing trend nowadays [119], however, it remains the fourth most common cancer and second leading cause of cancer-related death worldwide [76]. It is still an important medical problem and its prevention is one of the most important aspects of cancer control programs. Many pathological and biological analyses of gastric carcinomas, including precancerous lesions, have been performed with experimental animals as well as human samples. Major model animals susceptible to induction of gastric adenocarcinomas include rats, mice, and Mongolian gerbils, the latter two offering powerful tools for analysis of *Helicobacter pylori*-associated gastric disorders. In this article, we introduce major animal models while comparing with human lesions, concentrating special attention on pathological and biological findings.

H. pylori infection and the development of gastric cancer: epidemiological findings

H. pylori has been discovered from patients of chronic gastritis as gram-negative, flagellate, and microaerophilic

bacilli [52, 127] and has been revealed as a major causative factor for gastric disorders. Strong epidemiological evidence has been accumulated indicating a significant relationship with active chronic gastritis, peptic ulcers, atrophic gastritis, intestinal metaplasia, and malignant lymphoma or cancer development [3, 15, 18, 23, 28, 29, 42, 67, 77, 78, 114]. Prospective study confirmed that gastric cancers developed in 2.9 % of the *H. pylori*-infected group but none of the uninfected patients [126]. Based on the epidemiological findings, *H. pylori* was defined as a "definite biological carcinogen" by World Health Organization/International Agency for Research on Cancer (WHO/IARC) in 1994. However, regarding the relationship between *H. pylori* infection and development of gastric cancers, the lack of evidence of induction in experimental animals was one point, which received stress in the WHO/IARC report [30].

Establishment of the animal models

To identify pathological and molecular biochemical mechanisms, various experimental animal models have been established in rats and mice with chemical carcinogens including *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) and *N*-methyl-*N*-nitrosourea (MNU). Since *H. pylori* is one of the most important factors for human stomach disorders, the *H. pylori*-infected and carcinogen-treated Mongolian gerbil has proven very useful for analyses of underlying processes [123].

Rat models

In some of the earliest studies, researchers attempted to induce gastric cancers in animal models using chemical carcinogens such as benzo[a]pyrene [82] or 3-methylcholanthrene [90] by direct injection into the stomach or the gastric wall. Others tried oral administration, a more natural administrative method, using 2-acethylaminofluorene [129]. However, the resultant incidences of lesions were low. In 1967, however, Sugimura and Fujimura [92] utilized *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) in drinking water and were able to report good yields of adenocarcinomas in the glandular stomachs of rats. Upon exposure to MNNG, erosive lesions occurred and subsequent disordering of glandular structures and proliferation of epithelial cells in the pyloric mucosa were observed. After appearance of atypical glands, finally both differentiated and undifferentiated adenocarcinomas were induced in this model, mimicking the situation in humans. Oral administration of 4-nitroquinoline 1-oxide (4-NQO) and 4-hydroxyaminoquinoline 1-oxide (4-HAQO) similarly induces carcinomas in the stomach as well as the various other tissues [61, 62]. The presence of surfactants, such as alkylbenzenesulfonate, was found to enhance the effects of 4-NQO in the stomach of animals [96, 99]. Another carcinogen, *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine [43, 94], could be shown to cause gastric cancers not only in rats but also in dogs.

Mouse models

Administration of MNNG in the drinking water to BRSUNT/NJms mice over the life span only resulted in adenomatous hyperplasia of the gastric epithelium, suggesting resistance to this carcinogen [93]. However, oral administration of 4-NQO and 4-HAQO did induce carcinomas in the stomach as well as various other tissues [61, 62]. Finally, MNU was successfully introduced for good yields of adenocarcinomas in the glandular stomach of BALB/c mice [110]. Comparable yields were obtained in the C_3H [111] and other strains [133]. In the mouse models, both differentiated and undifferentiated types of adenocarcinomas typically develop, showing more significant cellular atypia compared with lesions in MNNG-treated rats. Taking advantage of this characteristic, intramucosal neoplasms became recognizable. The establishment of mouse models further opened up new approaches using transgenic and knockout animals. Yamamoto et al. utilized p53 knockout mice and revealed that nullizygous p53 knockout mice showed higher susceptibility to carcinogen in contrast to heterozygous animals [132].

Helicobacter-infected mouse models

H. pylori infection has been tried on many animals to study the pathogenetic background, but none of the early models proved sufficiently similar to the human situation [41, 80]. In 1990, Lee et al. [46] isolated *Helicobacter felis* from the cat stomach and inoculated germ-free mice with the bacteria, which colonized in the stomach like as *H. pylori* and induced acute and chronic inflammation. p53 wild-type and hemizygous mice were inoculated with *H. felis* and the latter showed higher proliferative index in the gastric foveolar epithelium [19]. *H. pylori* isolated from human clinical specimens were also utilized to inoculated nude and euthymic mice causing chronic active gastritis [34, 35, 51]. *H. pylori*, named Sydney strain (SS1), with higher colonizing ability were established by screening of fresh clinical isolates in long-term mouse adaptation and is currently widely used worldwide for mice experiments [47].

Oshima et al. constructed transgenic mice (K19-C2mE transgenic mouse) simultaneously expressing cyclooxygenase-2 (COX-2) and microsomal prostaglandin E synthase-1 under keratin 19 promoter in the gastric epithelial cells and revealed the importance of these pathways in gastric tumorigenesis especially with *Helicobacter* infection [72]. Additional expression of Wnt1 converted more dysplastic gastric tumors [73]. MNU treatment and *H. pylori* (Sydney strain, SS1) infection of K19-C2mE mice induced adenocarcinomas

not only in pyloric mucosa but also in fundic glands, serving a better model for increasing proximal gastric cancers [101].

H. pylori-infected Mongolian gerbil models: promoting effects of *H. pylori*

Besides the mouse models, Mongolian gerbil (*Meriones unguiculatus*) model was successfully established to mimic human severe *H. pylori* infection and inflammation, with the bacteria detectable throughout the study period up to 1 year [25]. Gerbils can be readily infected with *H. pylori*, and the resultant chronic active gastritis, peptic ulcers, and intestinal metaplasia resemble lesions apparent in man (Fig. 1a). Later in 1998, stomach carcinogenesis model was established using Mongolian gerbils with MNU and MNNG as the carcinogens (Fig. 1b) [112]. *H. pylori* infection was subsequently found to increase the incidence of both MNU- and MNNG- induced adenocarcinomas of all histological types including differentiated and undifferentiated adenocarcinomas and signet-ring cell carcinoma in the gerbils' glandular stomach (Fig. 1c, d) [85, 86, 95]. Several studies based on detailed histopathological assessment showed no carcinomas in animals treated only with *H. pylori* infection [85–87, 95, 112]. Thus, we consider that *H. pylori* is a strong promoter of gastric carcinogenesis. The *H. pylori*-infected and chemical carcinogen-treated Mongolian gerbils have thus proved very useful for the analysis of gastric carcinogenesis.

Besides adenocarcinomas, neuroendocrine tumors (NET) (endocrine cell hyperplasia/dysplasia and carcinoid tumors) were frequently induced in *H. pylori*-infected Mongolian gerbil model in association with serum gastrin level. Eradication of *H. pylori* prevents occurrence of NET lesions in the glandular stomach, this being strongly linked with reduction in serum gastrin levels [9]. Proton pump inhibitors (PPIs), routinely used for control of upper gastrointestinal disorders, have been some concern about the long-term safety and the possibility of cancer induction and development of NET in the stomach. When PPI was administered to *H. pylori*-infected Mongolian gerbils, PPI at high dose increased NET development with higher serum gastrin; in contrast, PPI at low dose had no influence on development of carcinomas and NETs in the *H. pylori*-infected and uninfected gerbils' glandular stomach [124].

Modifying factors of stomach carcinogenesis

Host risk factors for gastric cancer: age of infection of *H. pylori*

Childhood infection with *H. pylori* is a major concern in the pediatrics field. To compare severity of inflammation and susceptibility to induction of cancer in childhood and the adult, *H. pylori* inoculation and subsequent MNU administration were started at different time points in the gerbil's lifespan. Early acquisition of *H. pylori* significantly increased gastric carcinogenesis in carcinogen-treated gerbils, as compared to the case with later infection, possibly because of differences in host gastric mucosal and immunologic factors, as well as age-dependent sensitivity to chemical carcinogens (Fig. 1e, f) [5]. This would imply that childhood *H. pylori* infection must not be overlooked in

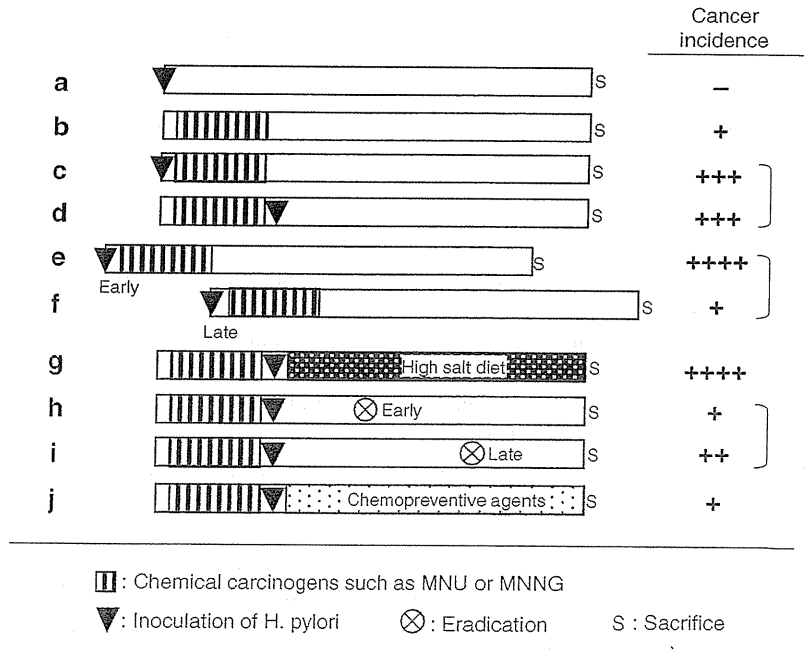

Fig. 1 Modifying factors for *H. pylori*-associated stomach carcinogenesis in Mongolian gerbils. **a** *H. pylori* infection alone is not carcinogenic. **b** Drinking water containing chemical carcinogens including MNU or MNNG induces stomach cancers. **c**, **d** When combined, *H. pylori* become a strong promoter. **e**, **f** Earlier infection increases the risk than later event. **g** A high-salt diet exacerbates inflammation and increases the incidence of *H. pylori*-associated cancer. **h**, **i** Earlier eradication of *H. pylori* reduces risk of stomach cancers. **j** Various natural products and pure chemicals appear to have chemopreventive potential

approaches to the prevention of gastric cancer later in adult life [14, 50].

Synergistic effects of *H. pylori* and high-salt diet

Among various food ingredients, salt and salted foods are probable risk factors for gastric cancer, based on evidence from a large number of case–control and ecological studies [33, 39, 97, 118]. In an experimental study, sodium chloride (NaCl) was found to enhance the carcinogenic effects of MNNG and 4-NQO in the rat glandular stomach [103], possibly partly due to decrease in the viscosity of the gastric mucus and so reduction in the protective mucous barrier. When given alone, NaCl has no apparent carcinogenicity in rats but, when administered with MNNG or 4-NQO, it promotes gastric carcinogenesis in the rat glandular stomach [103], in a dose-dependent fashion [100]. A high concentration causes initial tissue damage and consequent regenerative cell proliferation and this is also in line with accelerated lesion development [21].

Furthermore, in 2002, Nozaki et al. demonstrated a high-salt diet to enhance the effects of *H. pylori* infection on gastric carcinogenesis, with these two factors acting synergistically to promote the development of stomach cancers in the Mongolian gerbil model, although high-salt intake alone has a minor influence compared to *H. pylori* [68]. Further, to examine the dose dependence and the mechanisms underlying enhancing effects, Mongolian gerbils were treated with MNU, *H. pylori*, and food containing various concentrations of salt, and were sacrificed after 50 weeks. Among gerbils treated with MNU and *H. pylori*, the incidences of glandular stomach cancers were 15 % in the normal diet group and 33, 36, and 63 % in the 2.5, 5, and 10 % NaCl diet groups, showing dose-dependent increase. Intermittent intragastric injection of saturated NaCl solution, in contrast, did not promote gastric carcinogenesis. In gerbils infected with *H. pylori*, a high-salt diet was associated with elevation of anti-*H. pylori* antibody titers, serum gastrin levels, and inflammatory cell infiltration in a dose-dependent fashion (Fig. 1g). Ten percent NaCl diet upregulated the amount of surface mucous cell mucin, suitable for *H. pylori* colonization, despite no increment of MUC5AC mRNA, while *H. pylori* infection itself had an opposing effect, stimulating transcription of MUC6 and increasing the amount of gland mucous cell mucin. High-salt diet, in turn, decreased the amount of gland mucous cell mucin, which acts against *H. pylori* infection by inhibiting the bacterial cell wall component [38]. Reduction of salt intake could thus be one of the most important chemopreventive methods for human gastric carcinogenesis [36].

Besides the salt itself, *H. pylori* infection and high intake of various traditional salt-preserved foods are regarded as risk factors for human gastric cancer. Indole compounds, such as indole-3-acetonitrile is contained in Chinese cabbage and converted to a mutagen, 1-nitrosoindole-3-acetonitrile (NIAN), with nitrite under acidic condition in the stomach. Administration of NIAN to Mongolian gerbils induced well to moderately differentiated adenocarcinomas under *H. pylori* infection. Such lesions were not induced in gerbils given NIAN alone or infection with *H. pylori* alone. Thus, endogenous carcinogens formed from nitrosation of indole compounds could be critical risk factors for human gastric cancer development under the influence of *H. pylori* infection. [53]

Prevention of gastric cancer by eradication of *H. pylori*

To clarify the effects of eradication of *H. pylori* on prevention of gastric cancer development in patients with chronic gastritis, Uemura et al. [125] conducted a nonrandomized *H. pylori* eradication trial in cases whose gastric cancer was removed by endoscopic resection, and suggested that *H. pylori* eradication might improve neutrophil infiltration and intestinal metaplasia in the gastric mucosa and inhibit the development of new carcinomas. A randomized controlled trial conducted in China revealed that the incidence of gastric cancer development at the population level was similar between participants receiving *H. pylori* eradication treatment and those receiving placebo over a period of 7.5 years [130]. However, in the subgroup of *H. pylori* carriers without precancerous lesions, eradication of *H. pylori* significantly decreased the development of gastric cancer [130]. In *H. pylori*-infected Mongolian gerbils treated with MNU, Shimizu et al. have provided direct evidence that *H. pylori* eradication may be useful as a prevention approach against gastric cancer [87]. The incidences of gastric cancers after curative treatment for *H. pylori* were thus significantly lower than without *H. pylori* eradication. For further evaluation, an experimental model with eradication in the early, middle, and late periods was studied using *H. pylori*-infected and MNU-treated Mongolian gerbils [70]. *H. pylori* infection was found to strongly enhance gastric carcinogenesis initiated with the chemical carcinogen, and following eradication at an early period, this effect was effectively reduced (Fig. 1h, i).

Reversibility of heterotopic proliferative glands induced by *H. pylori* infection

As WHO/IARC has mentioned in 1994 [30], it had to be clarified whether or not *H. pylori* itself was a carcinogen. Several studies based on histopathology showed no carcinomas in animals treated only with *H. pylori* infection [85–87, 95, 112]. However, two reports concluded that *H. pylori* infection alone can induce well-differentiated adenocarcinomas at very high incidences in the glandular stomach

of Mongolian gerbils [27, 128], while another study resulted in only one poorly differentiated adenocarcinoma [26]. The incidences and histological patterns of the lesions differed greatly in these three reports. After *H. pylori* infection, glands in the stomach of gerbils start to proliferate into the submucosa, disrupting the lamina muscularis mucosa. Resultant lesions, termed heterotopic proliferative glands (HPGs), frequently develop with *H. pylori* infection in the glandular stomach of infected Mongolian gerbils, with minimal dysplastic change of constituent cells [69]. HPGs often resemble differentiated or mucinous adenocarcinomas showing structural abnormality, but lacked obvious cellular atypia. Their characteristics are: (1) organized polarity of their component cells; (2) differentiation from gastric phenotype HPGs into intestinal phenotype HPGs with mature Paneth cells; (3) formation of large cystic dilatations containing mucin, often with calcification; (4) shedding of epithelial cells and necrosis at the tips of lesions; (5) high-grade inflammation with infiltration of inflammatory cells (neutrophils in acute phase and mononuclear cells in chronic phase); and (6) organized polarity of proliferating zones (Table 1 and Fig. 2) [69]. These features are quite different from those of well-differentiated adenocarcinomas, which are characterized by obvious cellular atypia. After eradication, HPGs are obviously reduced, and gastric lesions in mucosa also disappear with little evidence persisting of the former injury. Reversible HPGs are induced solely by *H. pylori* infection in this species, and our studies have shown they are related to severe gastritis, rather than being malignant in character. Eradication of *H. pylori* induces apoptosis and suppresses proliferation in HPGs of infected Mongolian gerbils, these lesions thus being apparently reversible through regulation of cell kinetics [6]. Thus, distinguishing reversible lesions from true neoplasms is necessary in investigating the relationship of *H. pylori* infection with gastric carcinogenesis in the Mongolian gerbil model [69]. Taking into account all the available data, we conclude that *H. pylori* is a strong promoter of gastric carcinogenesis rather than an initiator.

Chemoprevention of gastric cancer

COX-2 inhibitor

Overexpression of COX-2 has been shown to be associated with several cancers, including gastric and colorectal adenocarcinomas in mice models [48, 72, 73, 79, 101] (Fig. 1j). Furthermore, COX-2 selective inhibitors such as etodolac and celecoxib may have chemopreventive effects [22, 49] not only suppressing inflammation, but also causing regression of early-stage tumors [10, 81]. Therefore, there is a possibility that COX-2 inhibitors could be useful drugs for regression of remaining precancerous lesion and prevention of gastric cancer occurrence after *H. pylori* eradication.

Considering the human situation, eradication of *H. pylori* significantly lowers the development of metachronous gastric cancer [20]. However, there has not been any approach to prevent gastric carcinogenesis in extensive metaplastic gastritis, an *H. pylori*-negative precancerous lesion. Forty-seven patients with extensive metaplastic gastritis were selected based on endoscopic findings and serum pepsinogen test-positive and *H. pylori* antibody-negative conditions. Nonrandomized etodolac treatment (300 mg/day) was administered to 26 patients, while the remaining 21 were untreated up to 5 years. Five cancers developed in non-treated group significantly more than one case in the etodolac group. Long-term etodolac treatment effectively reduced metachronous cancer development in patients with extensive metaplastic gastritis, while it did not influence the extent of metaplastic gastritis. Regulation of COX-2 could be an effective chemoprevention of gastric cancer in the metaplastic gastritis. [134]

Table 1 Histological difference of heterotopic proliferative glands and well-differentiated adenocarcinoma

	Heterotopic proliferative glands (HPGS)	Well-differentiated adenocarcinoma
Distribution of glands	Dispersed	Compactly proliferated
Shape of glands	Large and cystic	Relatively small
Intracystic material	Eosinophilic, sometimes with calcification	Usually transparent
Lining epithelium	Shedding of epithelial cells with necrosis at the tip of the cyst	Fully lined with atypical cells.
Intestinalization	Frequent, sometimes with Paneth cells	Relatively infrequent
Stroma	Severe inflammatory cell infiltrates. Neutrophils in acute phase. Lymphocytes and plasma cell in chronic phase with lymphoid follicle formation.	Desmoplastic reaction
Cellular component	No atypia. Organized polarity. Partly degenerated.	Enlarged nuclei with increased chromatin. Loss of polarity.

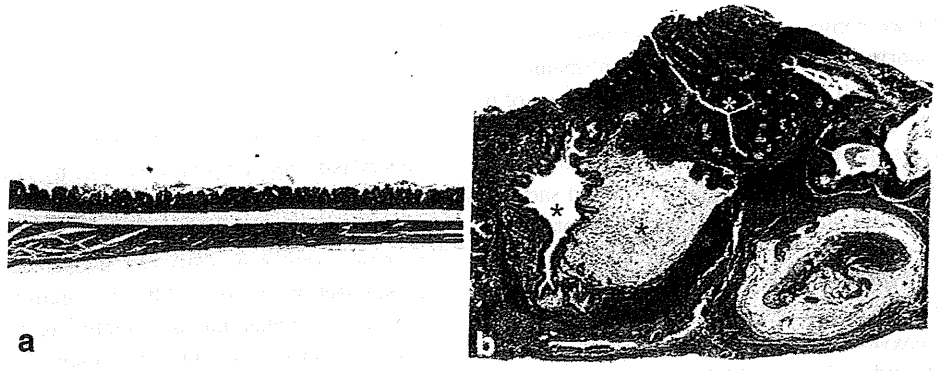

Fig. 2 Severe inflammatory response caused by long-term *H. pylori* infection in a Mongolian gerbil. **a** Normal gastric mucosa without *H. pylori* infection. **b** Heterotopic proliferative glands (HPGs) in the glandular stomach infected with *H. pylori* for 75 weeks. Proliferation and dilatation of gastric type gland (*red asterisk*) and intestinal metaplastic glands (*yellow asterisk*) with lymphoid follicle formation (*green asterisk*). Mucous lakes (*blue asterisks*) are developed with necrotic material (*blue asterisk on the right*). Hematoxylin and eosin staining

Oxygen radical scavenger

Recent epidemiological and experimental studies have demonstrated that consumption of certain natural products can lower gastric cancer risk in humans and animal models [120]. It has been also suggested that oxidative stress associated with inflammation plays an important role in gastric carcinogenesis as a mediator of DNA damage and carcinogenic compound formation [64]. Since the major determinant factor of gastric carcinogenesis is the severity of *H. pylori*-induced inflammation [7], the inhibition of *H. pylori*-induced inflammation and subsequent oxidative stress is a reasonable approach to prevent gastric cancer development.

To assess this hypothesis in the gerbil model, 4-vinyl-2,6-dimethoxyphenol (canolol), one of the most potent antioxidative compounds obtained from crude canola oil, was chosen to examine preventive effect of *H. pylori*-induced gastritis and gastric carcinogenesis [8]. The gerbils were subjected to *H. pylori*+MNU administration and were fed for 44 weeks with or without 0.1 % canolol. *H. pylori*-induced gastritis, expression of COX-2 and inducible nitric oxide synthase (iNOS), and increase in serum 8-hydroxy-2′-deoxyguanosine (8-OHdG) level were all attenuated in the canolol-treated groups. In addition, the incidence of gastric adenocarcinomas was markedly reduced. These data indicate that oxygen radical scavengers may suppress gastric inflammation and carcinogenesis in *H. pylori*-infected gerbils. Interestingly, the viable *H. pylori* count was not changed by the canolol-containing diet. Thus, the data point to the level of inflammation due to *H. pylori* rather than the existence of the bacteria as the determining factor is used.

Lignan, a plant-derived chemical

Another example of a chemopreventive agent is plant-derived lignan, a major group of plant compounds classified as phytoestrogens, which have attracted interest in recent years [1]. (+)-Syringarecinol, one of lignans contained in Japanese apricot, inhibited >90 % of the *H. pylori* motility at a concentration of 500 μg/mL and the IC50 value was 50 μg/mL *in vitro* [55]. Concentrated Japanese apricot significantly alleviated *H. pylori*-induced inflammation in Mongolian gerbils [75].

Since other lignans including arctiin, arctigenin, and nordihydroguaiaretic acid (NDGA) also inhibited proliferation and motility of *H. pylori in vitro* dose dependently, *in vivo* analyses were performed to assess preventive effect on *H. pylori*-associated gastritis and gastric cancer development in Mongolian gerbils using NDGA and arctigenin [115]. NDGA significantly decreased the incidence of gastric adenocarcinomas, the formation of intestinal metaplasia, and serum 8-OHdG levels. There were no differences in the titers of anti-*H. pylori* IgG or the expression of the *H. pylori*-specific *urease A* gene among all *H. pylori*-infected groups. These results suggest that NDGA might have suppressive effects on gastric carcinogenesis, with inhibitory effects on progression of gastritis and antioxidative activity rather than direct antimicrobial influence as the major mechanisms. Arctigenin, in contrast, failed to attenuate neoplasia in gerbils in spite of its potent suppressive effect on *H. pylori in vitro*. These results suggest the importance of *in vivo* animal experiments as well as *in vitro* analyses.

Nuclear factor-κB inhibitor

Nuclear factor-κB (NF-κB) plays a major role in host inflammatory responses and carcinogenesis and as such is an important drug target for adjuvant therapy. One of NF-κB inhibitors, caffeic acid phenethyl ester (CAPE), was analyzed on *H. pylori*-induced NF-κB activation in cell culture and chronic gastritis in Mongolian gerbils. In AGS human gastric cancer cells, CAPE significantly inhibited *H. pylori*-