TABLE III – INCIDENCE OF GASTRIC CARCINOMAS IN GERBILS

Experiments	Groups	No.	Treatments	Carcinoma		
				Dif.	Undif.	Incidence (%)
Experiment II	G	40	*Hp* + MNU->Canolol + BHT	5	1	6/40 (15.0)[1]
	H	33	*Hp* + MNU->BHT	11	2	13/33 (39.4)
	I	36	*Hp* + MNU	15	0	15/36 (41.7)
	J	5	Broth->Canolol + BHT	0	0	0/5 (0.0)

Dif., differentiated adenocarcinoma; Undif., undifferentiated adenocarcinoma. *Hp*, *H.pylori* (i.g.).
[1]$p = 0.031$ to Group H and $p = 0.011$ to Group I with Fisher's exact test.

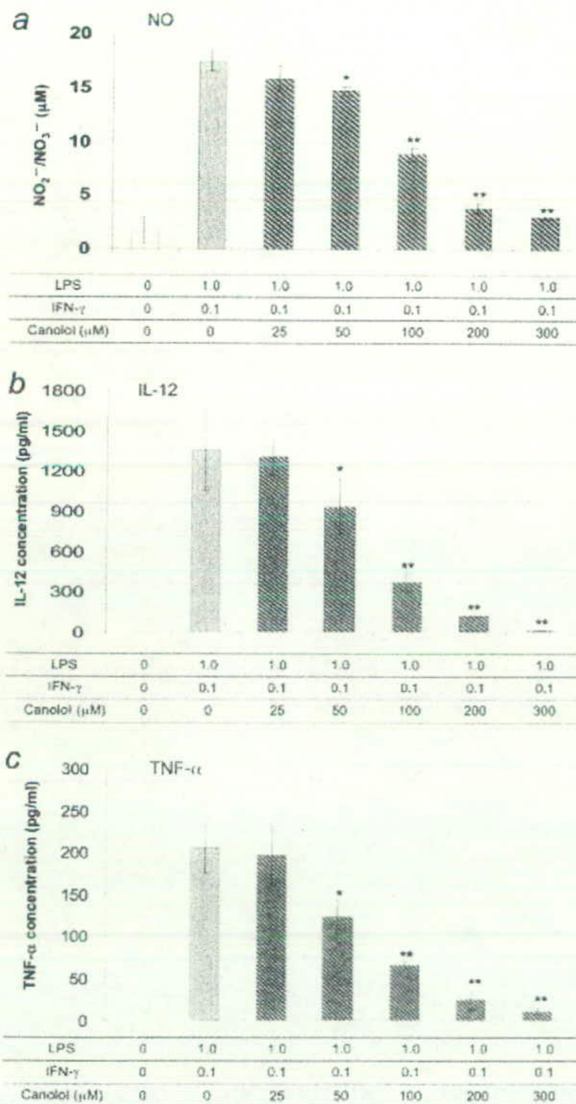

FIGURE 5 – Suppression of NO and inflammatory cytokine induction in mouse peritoneal macrophages *in vitro* by canolol. The concentrations of NO (*a*), IL-12 (*b*) and TNF-α (*c*), were significantly reduced by 12.5 μM canolol (means ± SD) ($n = 3$). $*p < 0.05$, $**p < 0.01$ *vs*. LPS plus IFN-γ.

were electrophoresed in 3% agarose gels and visualized with ethidium bromide after the LightCycler reaction. Quantification was performed as earlier established using an internal control without any necessity for external standards. The levels of cytokine mRNAs were expressed relative to 1.0 in the control groups (Groups F and J).[20]

*Elevation of 8-OHdG, anti-*H. pylori *IgG and gastrin in* H. pylori *infected gerbil plasma*

Before the removal of stomachs, blood samples were collected from the inferior vena cava after laparotomy. Sera were separated from blood and their anti-*H. pylori* IgG antibody titers were measured with an ELISA (GAP-IgG; Biomerica, Newport Beach, CA) and values expressed as an arbitrary index (AI). AI values of more than 1.5 indicated *H. pylori* infection. Gastrin levels were measured using a radioimmunoassay kit (SRL, Tokyo, Japan). Serum samples were also centrifuged (4°C, 10,000*g* for 30 min) through centrifugal filter devices (Microcon YM-10, Millipore, Bedford, MA) and measured for 8-OHdG levels (ELISA; high sensitive 8-OHdG check; Japan Institute for Control of Aging, Shizuoka, Japan).[21]

Statistical analyses

Quantitative values were expressed as means ± SD, and differences between means were evaluated by the Bonferroni multiple-comparison test. *p* values of less than 0.05 were considered significant.

Results

Canolol intake and bacterial colonization

The survival rates of all groups were >95%, with no differences among groups. In the shorter term experiment (Experiment I), total canolol intakes in Groups A and D were 0.45 ± 0.01 and 0.47 ± 0.01 (g/gerbil), respectively. There were no significant differences between Groups A and D. At 12 weeks postinfection, the numbers of *H. pylori* colonies were 3.17 ± 1.51, 3.52 ± 0.67 and 3.59 ± 0.85 ($\times 10^4$ colony/half stomach) in Groups A, B and C, respectively. No significant inhibitory effect of canolol against bacterial growth was detected ($p = 0.64$). In the longer term experiment (Experiment II), total canolol intakes in Groups G and J were 1.84 ± 0.02 and 1.84 ± 0.01 (g/gerbil), respectively, again with no significance. There was also no significant variation in body weights (Supplementary Fig. 1) among long-term experiment groups G to J, confirming no apparent toxicity of canolol.

Effects of canolol against H. pylori*-induced gastritis and cell proliferation*

All gastric mucosal specimens from uninfected gerbils had normal histomorphology. The histological findings for gastric mucosal specimens in *H. pylori*-infected gerbils are shown in Table II. At 12 weeks (Experiment I), neutrophils and lymphoplasmocytic cell infiltration in the antral mucosa of the canolol-treated group (Group A) were significantly suppressed, compared to the *H. pylori*-infected control groups (Groups B and C) (Table II; Figs. 3*a* and 3*b*). Both antral and corpus BrdU labeling indices in the canolol-treated gerbils (Group A) were significantly reduced as compared to values for control groups B and C ($p < 0.05$) (Table II). The BrdU LIs in Group A were reduced to 62% in the antrum and 71% in the corpus of the Group B values. During the 52 weeks (Experiment II) there was a change over time in topography of the gastritis, with a shift from predominantly antral gastritis to pangastritis in *H. pylori*-infected gerbils. Infiltration of inflammatory

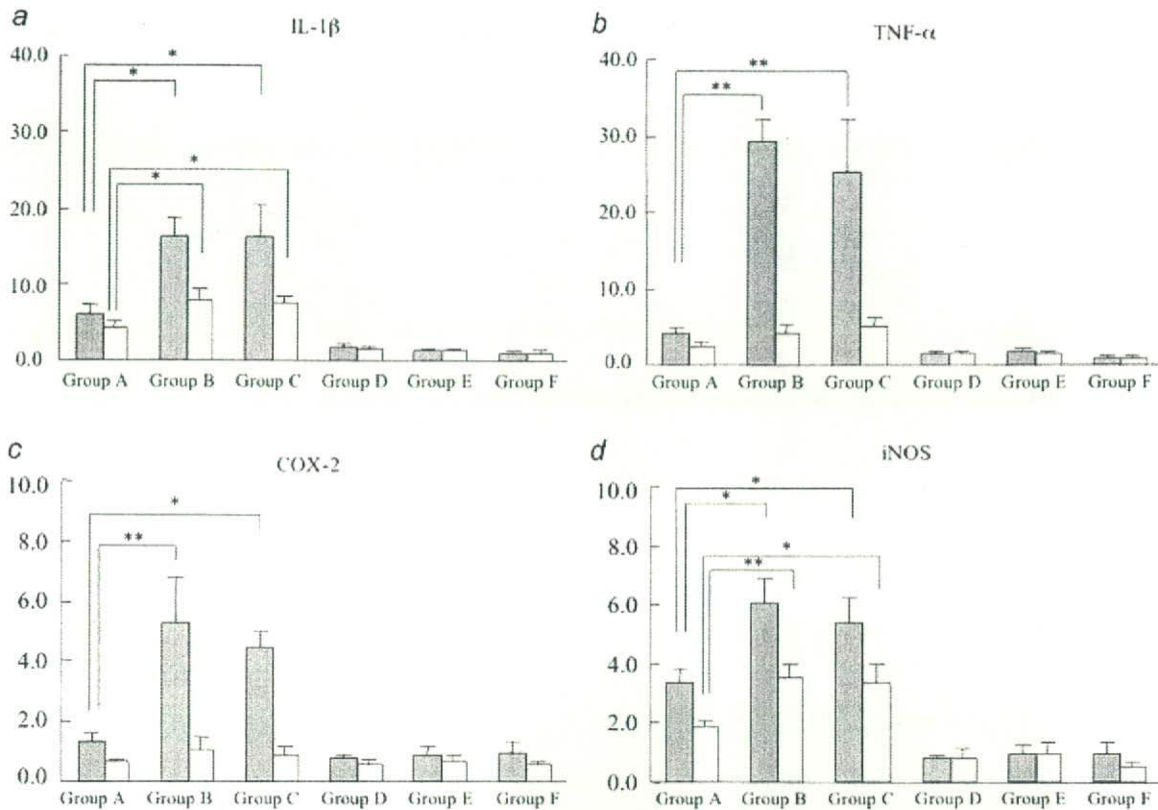

FIGURE 6 – Relative expression levels of IL-1β, TNF-α, COX-2, and iNOS mRNAs in glandular stomachs of gerbils at 12 weeks postinfection. (*a*) IL-1β; (*b*) TNF-α; (*c*) COX-2; (*d*) iNOS. Values are arbitrary unit values (mean ± SE) relative to 1.0 for controls. Note decrease in Group A (canolol group) as compared to Groups B and C (controls), especially in the antrum. ▨, Antrum; □, corpus. $*p < 0.05$ and $**p < 0.01$.

cells, hyperplasia and intestinal metaplasia lesions of gastric mucosa were markedly lower in the canolol-treated group (Group G) than in the *H. pylori*-infected control groups (Groups H and I). BrdU LIs in the canolol-treated gerbils (Group G) were again significantly lower both in antrum and corpus than in the control groups H and I, values being decreased to 56 and 64% of the Group H levels ($p < 0.05$) (Table II).

Immunohistochemistry of COX-2 and iNOS

Immunoreactivity against COX-2 and iNOS was evident in all *H. pylori*-infected gerbils. However, scores in the canolol-treated groups were significantly lower than in the canolol-untreated control groups (Fig. 4 and Table II).

Canolol suppression of gastric carcinogenesis

In Experiment II, the incidence of glandular stomach tumors overall was significantly lower in Group G (*H. pylori* + MNU + canolol + BHT) compared to Groups H (*H. pylori* + MNU + BHT) [15.0% (6/40) *vs.* 39.4% (13/33), $p = 0.031$] and I (*H. pylori* + MNU) [15.0% (6/40) *vs.* 41.7% (15/36), $p = 0.011$] at 52 weeks postinfection (Table III; Figs. 3*c* and 3*d*). There was no difference in the incidence of gastric adenocarcinomas between Groups H and I [39.4% (13/33) *vs.* 41.7% (15/36), $p = 1.00$]. In the control group J and Experiment I, no tumors developed in the glandular stomach.

Suppression of NO and inflammatory cytokines by canolol in mouse peritoneal macrophages in vitro

LPS and IFN-γ induction of NO, IL-12 and TNF-α was significantly inhibited by 50 μM canolol or above *in vitro* (Figs. 5*a*–5*c*).

Oral administration of canolol and mRNA expression of IL-1β, TNF-α, COX-2 and iNOS

Gastric IL-1β, TNF-α, COX-2 and iNOS were found to be expressed at very low levels in the uninfected control gerbils. However, in the *H. pylori*-infected groups, the levels of these cytokines and enzymes were markedly elevated in the antrum and corpus already 12 weeks after infection (Experiment I, Fig. 6). Relative expression of IL-1β (Fig. 6*a*) in Groups B and C was upregulated 16 ± 2 and 16 ± 4 times, respectively, compared to the uninfected group F (control, its value set at 1.0 ± 0.2). Canolol treatment at 0.1% in the diet (Group A) significantly attenuated the increase of mRNA expression to 6.2 ± 1.1 times in the antrum ($p < 0.05$). For the corpus, it was elevated to 8.0 ± 1.3 (Group B) and 7.6 ± 0.7 (Group C) times with *H. pylori* infection and decreased to 4.3 ± 0.9 times with canolol treatment (Group A) ($p < 0.05$) compared to Group F (0.86 ± 0.15 times to corpus of Group F). The figures in Groups D, E and F were comparable. Regarding the expression of TNF-α (Fig. 6*b*), transcriptional upregulation 29 ± 3 and 25 ± 7 fold in *H. pylori* infected groups B and C, respectively, was alleviated to 4.0 ± 0.6 times in Group A ($p < 0.01$). Concerning Cox-2 expression (Fig. 6*c*), the figures were elevated to 5.3 ± 1.5 ($p < 0.01$) and 4.5 ± 0.5 ($p < 0.05$) times in Groups B and C, respectively, and decreased to 1.4 ± 0.7 times in Group A. For iNOS (Fig. 6*d*), the values were 6.1 ± 0.8 (Group B) and 5.4 ± 0.9 (Group C) times and lowered to 3.4 ± 0.4 times (Group A) in the antrum and 3.6 ± 0.4, 3.4 ± 0.6 and 1.8 ± 0.2 times in the corpus, respectively.

In Experiment II (Fig. 7), transcription of the inflammatory cytokines reached much higher levels than with the shorter experimental period. IL-1β mRNA was strongly upregulated 116 ± 17 (Group H) and 119 ± 23 (Group I) fold with long term *H. pylori*

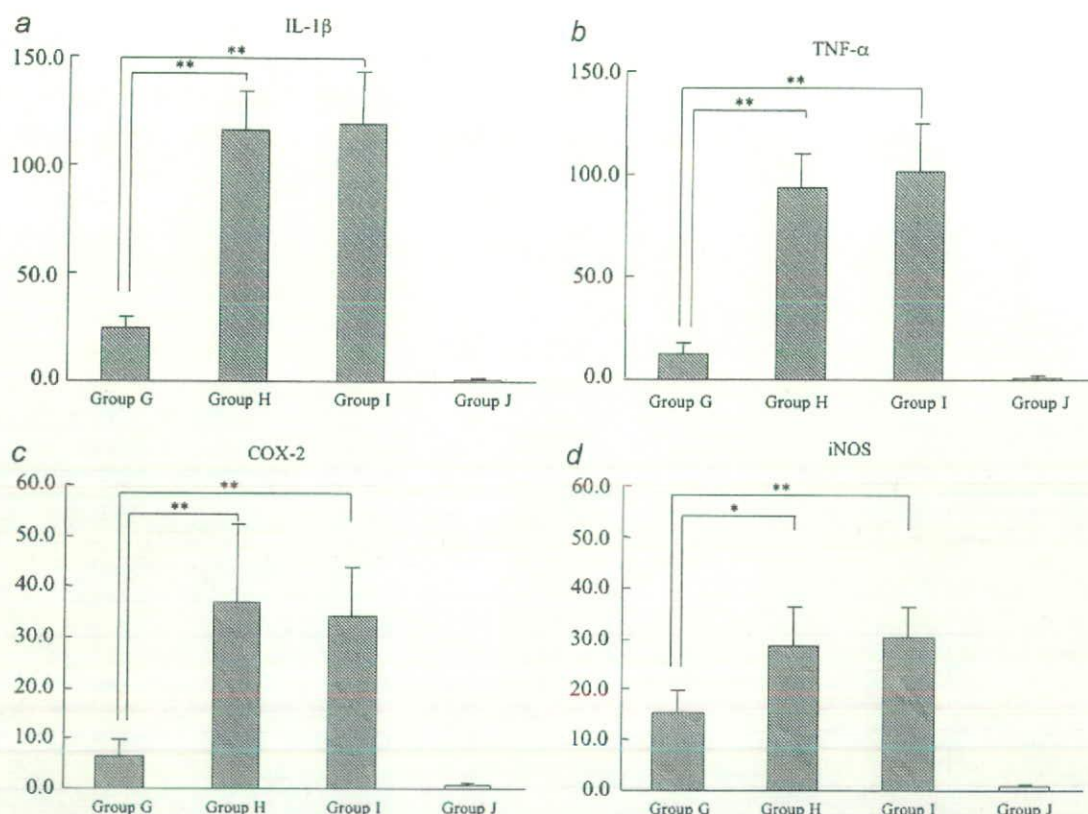

FIGURE 7 – Relative expression levels of IL-1β, TNF-α, COX-2 and iNOS mRNAs in glandular stomachs of gerbils at 52 weeks postinfection. (*a*) IL-1β; (*b*) TNF-α; (*c*) COX-2; (*d*) iNOS. Values are arbitrary unit values (mean ± SE) relative to 1.0 for controls. Note decrease in Group G as compared to Groups H and I. ▩, glandular stomach mucosa at the border between the antrum and corpus. $*p < 0.05$ and $**p < 0.01$.

TABLE IV – SERUM 8-OHDG, ANTI-*H.pylori* IGG TITERS AND GASTRIN LEVELS

Experiments	Groups	No.	Treatments	8-OHdG (ng/ml)	Antibody titers (A.I.)	Gastrin (pg/ml)
Experiment I	A	10	*Hp* -> Canolol + BHT	0.33 ± 0.05^1	19.6 ± 5.5^2	ND
	B	10	*Hp* -> BHT	0.48 ± 0.12	29.8 ± 7.6	ND
	C	10	*Hp*	0.51 ± 0.19	30.5 ± 8.2	ND
	D	10	Broth -> Canolol + BHT	0.30 ± 0.05	1.0 ± 0.4	ND
	E	10	Broth -> BHT	0.27 ± 0.05	1.4 ± 0.5	ND
	F	8	Broth	0.26 ± 0.07	1.1 ± 0.5	ND
Experiment II	G	40	*Hp* + MNU -> Canolol + BHT	0.41 ± 0.04^3	186.4 ± 74.2^4	634.0 ± 160.7^5
	H	33	*Hp* + MNU -> BHT	0.57 ± 0.07	249.5 ± 98.5	780.7 ± 216.2
	I	36	*Hp* + MNU	0.63 ± 0.12	257.9 ± 95.1	764.1 ± 195.7
	J	5	Broth -> Canolol + BHT	0.29 ± 0.09	1.1 ± 0.3	202.2 ± 54.4

8-OHdG, 8-hydroxy-2′-deoxyguanosine. ND, not determined. Values for results are expressed as means ± SD.
[1]$p < 0.05$ *vs.* Groups B and C.–[2]$p < 0.01$ *vs.* Groups B and C.–[3]$p < 0.01$ *vs.* Groups H and I.–[4]$p < 0.05$ *vs.* Groups H and I.–[5]$p < 0.05$ *vs.* Groups H and I.

infection and this was drastically attenuated to 25 ± 6 times with canolol treatment (Group G, $p < 0.01$ *vs.* Groups H and I). TNF-α transcription also increased 93 ± 17 and 101 ± 23 times in Groups H and I and reduced to 13 ± 5 times in Group G ($p < 0.01$). Regarding Cox-2, the figures of 37 ± 15 and 34 ± 10 times (Groups H and I) were decreased to 6.3 ± 3.2 times (Group G, $p < 0.01$). Finally, the values of iNOS (28 ± 8 and 30 ± 6 times in Groups H and I, $p < 0.05$ and $p < 0.01$, respectively) were again lowered to 15 ± 5 times (Group G).

*Effects of canolol on serum 8-OHdG, anti-*H. pylori *antibodies and gastrin levels*

Infection with *H. pylori* remarkably elevated the serum level of 8-OHdG and anti-*H. pylori* IgG titers in both Experiments I and II. Significant reduction was noted with canolol treatment (Table IV). After *H. pylori* infection, serum gastrin levels in Experiment II were elevated at 52 weeks, and this increase was alleviated in the canolol-treated group G (Table IV).

Discussion

Triple therapy consisting with a proton pump inhibitor and 2 antimicrobial agents, amoxicillin and clarithromycin, is usually recommended as the general therapy for *H. pylori* eradication in Japan.[22] However, frequent emergence of resistant strains to these antimicrobial agents, and persistence of gastric inflammation even after the eradication of *H. pylori*, has been observed by

physicians. Therefore we need to find means to attenuate gastric inflammation and provide cytoprotection against *H. pylori*-induced cytotoxicity.[23]

Our study showed *H. pylori*-associated chronic active gastritis and gastric carcinogenesis to be effectively suppressed by oral administration of canolol at 0.1% in the diet. In addition, iNOS, COX-2, and inflammatory cytokine IL-1β, IL-12 and TNF-α mRNA expression levels were substantially decreased after canolol administration *in vivo* and *in vitro*. It has been reported that a predominantly *H. pylori*-specific Th1 response, characterized by induction of high level of TNF-α, IL-1β, and IFN-γ is associated with *H. pylori*-infected gastritis.[24,25] COX-2 and iNOS are well known to play important roles in gastric cancer growth and progression. These results indicate that canolol inhibits the mRNA expression of COX-2, upregulated by *H. pylori*-infection, and might reduce release of prostaglandin E2 from the gastric mucosa.[26] Canolol also suppressed iNOS activity and presumably the NO endogenously produced by this family of enzymes. It is interesting to note that the numbers of *H. pylori* colonies in the glandular stomach at 12 weeks postinfection was not significantly reduced. Thus, suppression of IL-1β, TNF-α, COX-2 and iNOS, activated by *H. pylori*-infection, appears critical for the inhibition of gastric carcinogenesis. Crabtree *et al.*[27] showed an increase in the production of TNF-α in antral biopsy specimens from patients with *H. pylori* gastritis coinciding with neutrophil infiltration. Similarly, Harris *et al.*[28] described the number of mRNA molecules for IL-6 to be elevated to a greater extent in persistently infected rhesus monkeys (6 years) compared to the early phase (7 weeks after infection), whereas expression of IL-1β and TNF-α declined. However, gastric biopsies from persistently infected animals only showed weak gastritis. Yamaoka *et al.*[25] have reported natural history of *H. pylori* (ATCC43504) induced gastritis and associated gastric mucosal cytokine expression in Mongolian gerbils. In their results, polymorphonuclear and mononuclear cell infiltration was apparent relatively early (8 and 4 weeks after inoculation, respectively) and declined thereafter. Levels of cytokines, including IL-1β, INF-γ, IL-4, IL-6 and IL-10, appeared to be mostly parallel; the values for IFN-γ correlated particularly well with numbers of both polymorphonuclear and mononuclear cells. However, in our experiment, inflammation induced cytokine overexpression was greater in the long-term experiment compared to the short one (Fig. 7 *vs*. Fig. 6). In contrast to the data by Yamaoka *et al.*,[25] infiltration of the inflammatory cells progressively increased. Thus, in terms of the correlation of inflammatory cell infiltrate and cytokine expression level, data in our experiment and their results do not appear to be incompatible. *H. pylori* inoculated in gerbils might have been partially eradicated or their virulence could have become attenuated in their system.

Of note, at 12 and 52 weeks postinfection, BrdU-labeled cells in gastric mucosa decreased almost 50–70% in canolol-treated gerbils compared to those in *H. pylori*-infected control groups. Gonzalez *et al.* found a similar reduction of proliferating cells in ultraviolet B (UVB) exposed mouse epidermis with oral administration of antioxidants like lutein + zexanthin.[29] Kim *et al.* also observed lowering of BrdU LIs with carotenoids (lycopene, fucoxanthin and lutein) and curcumin and its derivative (tetrahydrocurcumin) in 1,2-dimethylhydrazine treated mouse colonic crypts, along with reduced aberrant crypt formation.[30] At the molecular level, reactive oxygen intermediates may alter the expression and function of Cox-2 and iNOS, which may influence the expression of proteins involved in regulation of cell cycle progression.[31] Similarly, anti-inflammatory and antioxidant agents could protect against such effects and also act on the expression and function of several cell cycle regulating proteins.[32]

Furthermore, the remarkable elevation of serum 8-OHdG by *H. pylori*-infection was alleviated by canolol. 8-OHdG has been proposed as a key biomarker of oxidative DNA damage relevant to carcinogenesis,[33] because of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), superoxide anions (O_2), singlet oxygen and hydroxyl radicals ($^{\bullet}OH$) as well as reactive nitrogen species (RNS) including $ONOO^-$, which is known to cleave DNA and also nitrate guanine to generate 8-nitroguanine. Mutation of *Salmonella sp.* (TA98) by $ONOO^-$ was earlier found to be effectively suppressed by canolol,[13] one of the most potent anti-$ROO^{\bullet}$ antioxidants.[13,34,35] Therefore, one of the underlying mechanisms is reduction of free radical scavenging oxidative damage.[36] In an *in vitro* system we could also show that canolol suppresses inflammation mediators (Fig. 5).

In conclusion, oral administration of canolol significantly reduced anti-*H. pylori* IgG antibody titers and gastrin levels in serum, without apparently suppressing *H. pylori* colonization. A lack of any direct correlation between anti-*H. pylori* IgG antibody titers and number of colonies was also reported by Murakami *et al.*[37] Canola oil is a traditional cooking oil in many countries. The canolol concentration in crude canola oil is estimated to be ~220–1,200 ppm, which could provide doses similar to that used in our study. It should be noted, however, that the concentration in refined canola oil is significantly lower[12] so that suggestions to the edible oil industry for alternative methods of refining might be warranted. Alternatively, synthesized or extracted canolol could be added to the refined oil and used as table oil or taken as a supplement. Taken together, these findings indicate that the antioxidative compound, canolol, can prevent *H. pylori*-induced gastritis and carcinogenesis in a gerbil model. Therefore, this dietary factor may have a potential role in controlling *H. pylori*-associated gastroduodenal diseases.

Acknowledgements

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References

1. Parsonnet J, Friedman GD, Vandersteen DP, Chang Y, Vogelman JH, Orentreich N, Sibley RK. *Helicobacter pylori* infection and the risk of gastric carcinoma. N Engl J Med 1991;325:1127–31.
2. Uemura N, Okamoto S, Yamamoto S, Matsumura N, Yamaguchi S, Yamakido M, Taniyama K, Sasaki N, Schlemper RJ. *Helicobacter pylori* infection and the development of gastric cancer. N Engl J Med 2001;345:784–9.
3. Danese S, Papa A, Gasbarrini A, Ricci R, Maggiano N. *Helicobacter pylori* eradication down-regulates matrix metalloproteinase-9 expression in chronic gastritis and gastric ulcer. Gastroenterology 2004;126: 369–71.
4. Wong BC, Lam SK, Wong WM, Chen JS, Zheng TT, Feng RE, Lai KC, Hu WH, Yuen ST, Leung SY, Fong DY, Ho J, et al. *Helicobacter pylori* eradication to prevent gastric cancer in a high-risk region of China: a randomized controlled trial. JAMA 2004;291: 187–94.
5. Graham DY. Antibiotic resistance in *Helicobacter pylori*: implications for therapy. Gastroenterology 1998;115:1272–7.
6. Matsubara S, Shibata H, Ishikawa F, Yokokura T, Takahashi M, Sugimura T, Wakabayashi K. Suppression of *Helicobacter pylori*-induced gastritis by green tea extract in Mongolian gerbils. Biochem Biophys Res Commun 2003;310:715–19.
7. Iimuro M, Shibata H, Kawamori T, Matsumoto T, Arakawa T, Sugimura T, Wakabayashi K. Suppressive effects of garlic extract on *Helicobacter pylori*-induced gastritis in Mongolian gerbils. Cancer Lett 2002;187:61–8.
8. Otsuka T, Tsukamoto T, Tanaka H, Inada K, Utsunomiya H, Mizoshita T, Kumagai T, Katsuyama T, Miki K, Tatematsu M. 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.
9. Kim H, Seo JY, Kim KH. Effect of mannitol on *Helicobacter pylori*-induced cyclooxygenase-2 expression in gastric epithelial AGS cells. Pharmacology 2002;66:182–9.
10. Correa P, Fontham ET, Bravo JC, Bravo LE, Ruiz B, Zarama G, Realpe JL, Malcom GT, Li D, Johnson WD, Mera R. Chemoprevention of gas-

tric dysplasia: randomized trial of antioxidant supplements and anti-*Helicobacter pylori* therapy. J Natl Cancer Inst 2000;92: 1881–8.
11. Sun YQ, Girgensone I, Leanderson P, Petersson F, Borch K. Effects of antioxidant vitamin supplements on *Helicobacter pylori*-induced gastritis in Mongolian gerbils. Helicobacter 2005;10:33–42.
12. Wakamatsu D, Morimura S, Sawa T, Kida K, Nakai C, Maeda H. Isolation, identification, and structure of a potent alkyl-peroxyl radical scavenger in crude canola oil, canolol. Biosci Biotechnol Biochem 2005;69:1568–74.
13. Kuwahara H, Kanazawa A, Wakamatu D, Morimura S, Kida K, Akaike T, Maeda H. Antioxidative and antimutagenic activities of 4-vinyl-2,6-dimethoxyphenol (canolol) isolated from canola oil. J Agric Food Chem 2004;52:4380–7.
14. Tatematsu M, Tsukamoto T, Inada K. Stem cells and gastric cancer—role of gastric and intestinal mixed intestinal metaplasia. Cancer Sci 2003;94:135–41.
15. Newmark HL, Huang MT, Reddy BS. Mixed tocopherols inhibit azoxymethane-induced aberrant crypt foci in rats. Nutr Cancer 2006;56:82–5.
16. Hirose M, Nishikawa A, Shibutani M, Imai T, Shirai T. Chemoprevention of heterocyclic amine-induced mammary carcinogenesis in rats. Environ Mol Mutagen 2002;39:271–8.
17. Ikeno T, Ota H, Sugiyama A, Ishida K, Katsuyama T, Genta RM, Kawasaki S. *Helicobacter pylori*-induced chronic active gastritis, intestinal metaplasia, and gastric ulcer in Mongolian gerbils. Am J Pathol 1999;154:951–60.
18. Tanaka T, Kohno H, Suzuki R, Hata K, Sugie S, Niho N, Sakano K, Takahashi M, Wakabayashi K. Dextran sodium sulfate strongly promotes colorectal carcinogenesis in Apc(Min/+) mice: inflammatory stimuli by dextran sodium sulfate results in development of multiple colonic neoplasms. Int J Cancer 2006;118:25–34.
19. Zingarelli B, Szabo C, Salzman AL. Reduced oxidative and nitrosative damage in murine experimental colitis in the absence of inducible nitric oxide synthase. Gut 1999;45:199–209.
20. Tsukamoto T, Fukami H, Yamanaka S, Yamaguchi A, Nakanishi H, Sakai H, Aoki I, Tatematsu M. Hexosaminidase-altered aberrant crypts, carrying decreased hexosaminidase a and b subunit mRNAs, in colon of 1,2-dimethylhydrazine-treated rats. Jpn J Cancer Res 2001;92:109–18.
21. Magari H, Shimizu Y, Inada K, Enomoto S, Tomeki T, Yanaoka K, Tamai H, Arii K, Nakata H, Oka M, Utsunomiya H, Tsutsumi Y, et al. Inhibitory effect of etodolac, a selective cyclooxygenase-2 inhibitor, on stomach carcinogenesis in *Helicobacter pylori*-infected Mongolian gerbils. Biochem Biophys Res Commun 2005;334:606–12.
22. Malfertheiner P, Megraud F, O'Morain C, Bell D, Bianchi Porro G, Deltenre M, Forman D, Gasbarrini G, Jaup B, Misiewicz JJ, Pajares J, Quina M, et al. Current European concepts in the management of *Helicobacter pylori* infection—the Maastricht Consensus Report. The European *Helicobacter pylori* Study Group (EHPSG). Eur J Gastroenterol Hepatol 1997;9:1–2.
23. Wang WH, Wong BC, Mukhopadhyay AK, Berg DE, Cho CH, Lai KC, Hu WH, Fung FM, Hui WM, Lam SK. High prevalence of *Helicobacter pylori* infection with dual resistance to metronidazole and clarithromycin in Hong Kong. Aliment Pharmacol Ther 2000;14:901–10.
24. D'Elios MM, Manghetti M, De Carli M, Costa F, Baldari CT, Burroni D, Telford JL, Romagnani S, Del Prete G. 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.
25. Yamaoka Y, Yamauchi K, Ota H, Sugiyama A, Ishizone S, Graham DY, Maruta F, Murakami M, Katsuyama T. Natural history of gastric mucosal cytokine expression in *Helicobacter pylori* gastritis in Mongolian gerbils. Infect Immun 2005;73:2205–12.
26. Romano M, Ricci V, Memoli A, Tuccillo C, Di Popolo A, Sommi P, Acquaviva AM, Del Vecchio Blanco C, Bruni CB, Zarrilli R. *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.
27. Crabtree JE, Shallcross TM, Heatley RV, Wyatt JI. Mucosal tumour necrosis factor a and interleukin-6 in patients with *Helicobacter pylori* associated gastritis. Gut 1991;32:1473–7.
28. Harris PR, Smythies LE, Smith PD, Dubois A. Inflammatory cytokine mRNA expression during early and persistent *Helicobacter pylori* infection in nonhuman primates. J Infect Dis 2000;181:783–6.
29. Gonzalez S, Astner S, An W, Goukassian D, Pathak MA. Dietary lutein/zeaxanthin decreases ultraviolet B-induced epidermal hyperproliferation and acute inflammation in hairless mice. J Invest Dermatol 2003;121:399–405.
30. Kim JM, Araki S, Kim DJ, Park CB, Takasuka N, Baba-Toriyama H, Ota T, Nir Z, Khachik F, Shimidzu N, Tanaka Y, Osawa T, et al. Chemopreventive effects of carotenoids and curcumins on mouse colon carcinogenesis after 1,2-dimethylhydrazine initiation. Carcinogenesis 1998;19:81–5.
31. Shackelford RE, Kaufmann WK, Paules RS. Oxidative stress and cell cycle checkpoint function. Free Radic Biol Med 2000;28:1387–404.
32. Baldassarre G, Nicoloso MS, Schiappacassi M, Chimienti E, Belletti B. Linking inflammation to cell cycle progression. Curr Pharm Des 2004;10:1653–66.
33. Kasai H. Analysis of a form of oxidative DNA damage, 8-hydroxy-2′-deoxyguanosine, as a marker of cellular oxidative stress during carcinogenesis. Mutat Res 1997;387:147–63.
34. Akaike T, Ijiri S, Sato K, Katsuki T, Maeda H. Determination of peroxyl radical-scavenging activity in food by using bactericidal action of alkyl peroxyl radical. J Agric Food Chem 1995;4:1864–70.
35. Kanazawa A, Sawa T, Akaik T, Maeda H. Formation of abasic sites in DNA by t-butyl peroxyl radicals: implication for potent genotoxicity of lipid peroxyl radicals. Cancer Lett 2000;156:51–5.
36. Okazaki K, Ishii Y, Kitamura Y, Maruyama S, Umemura T, Miyauchi M, Yamagishi M, Imazawa T, Nishikawa A, Yoshimura Y, Nakazawa H, Hirose M. Dose-dependent promotion of rat forestomach carcinogenesis by combined treatment with sodium nitrite and ascorbic acid after initiation with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine: possible contribution of nitric oxide-associated oxidative DNA damage. Cancer Sci 2006;97:175–82.
37. Murakami M, Ota H, Sugiyama A, Ishizone S, Maruta F, Akita N, Okimura Y, Kumagai T, Jo M, Tokuyama T. Suppressive effect of rice extract on *Helicobacter pylori* infection in a Mongolian gerbil model. J Gastroenterol 2005;40:459–66.

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Cellular and Molecular Biology

Synergistic upregulation of inducible nitric oxide synthase and cyclooxygenase-2 in gastric mucosa of Mongolian gerbils by a high-salt diet and *Helicobacter pylori* infection

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Summary. Aims: The intake of salt and salty food is known as a risk factor for gastric cancer. We have previously demonstrated that a high-salt diet dose-dependently enhances *Helicobacter pylori* (*H. pylori*)-associated gastritis and stomach carcinogenesis in Mongolian gerbils. In this study, we focused on the influence of excessive salt intake on the expression of inflammatory mediators involved in progression of *H. pylori*-induced chronic gastritis.

Methods and Results: A total of 45 stomach samples from Mongolian gerbils were evaluated by immunohistochemistry. The animals were infected with *H. pylori* and fed basal (0.32%) or a high-salt (10%) diet, and sacrificed after 40 weeks. Proliferative activity and expression of cyclooxygenase-2 (COX-2) in gastric mucosa were significantly increased in *H. pylori*-infected gerbils. The additional high-salt diet significantly up-regulated the expression of inducible nitric oxide synthase (iNOS) and COX-2 in *H. pylori*-infected groups ($P<0.01$ and $P<0.05$, respectively), while no significant effects were noted in non-infected animals. There was significant synergistic interaction between *H. pylori* infection and 10% NaCl diet on the expression of iNOS ($P<0.05$) and also a tendency for enhanced COX-2 expression ($P=0.0599$).

Conclusions: The present results suggest that a high-salt diet works synergistically with *H. pylori* infection to enhance iNOS and COX-2 expression in the gastric mucosa of Mongolian gerbils, and support the hypothesis that excessive salt intake may be associated with progression of *H. pylori*-induced gastritis.

Key words: Salt, Gastritis, *Helicobacter pylori*, iNOS, COX-2

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Introduction

Helicobacter pylori (H. pylori) is a major causative factor for gastric disorders and epidemiological evidence has accumulated indicating a significant relationship with gastric cancer development (Marshall and Warren, 1984; Uemura et al., 2001). In 1994, the World Health Organization/International Agency for Research on Cancer concluded that *H. pylori* is a "definite carcinogen" based on the epidemiological findings (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 1994). Recently, the concept that inflammation is a critical component of tumor progression has received a great deal of attention (Coussens and Werb, 2002). It is now known that there is a strong association between *H. pylori*-induced chronic atrophic gastritis and development of gastric cancer (Correa, 1995). Mongolian gerbils can readily be infected with *H. pylori,* and the resultant chronic active gastritis, peptic ulcers and intestinal metaplasia resemble lesions also apparent in humans (Hirayama et al., 1996; Sugiyama et al., 1998). We have previously reported that the severity of gastritis plays an important role in *H. pylori*-associated gastric carcinogenesis in gerbils, with essential involvement of chronic inflammation and increased rates of cell proliferation (Cao et al., 2007). Thus, investigation of the progression mechanisms of gastritis and the search for crucial factors for chemoprevention of gastric cancer continues to be very important.

Environmental and host factors are also known to influence gastric carcinogenesis, and salt (sodium chloride, NaCl) and salty foods are probably of particular importance, based on evidence from a large number of case-control and other epidemiological studies (Joossens et al., 1996; Kono and Hirohata, 1996; Tsugane, 2005). In Japan, foods containing salt at concentrations up to 12% are commonly consumed, such as pickled vegetables (salt content: 1-10%) and salted fish roe or fish preserves (6-12%) (Tsugane et al., 2004), and it has been reported that restriction of salty food

intake may decrease the risk of gastric cancer (Tajima and Tominaga, 1985; Shikata et al., 2006). In addition, several studies in mice and gerbils indicate that chronic excessive salt in the diet exerts synergistic effects with *H. pylori* infection on progression of gastritis and mucosal hyperplasia, also enhancing *H. pylori* colonization (Fox et al., 1999; Gamboa-Dominguez et al., 2007). Thus, the association between *H. pylori* and NaCl appears to be important for the progression of gastritis and the associated carcinogenesis, although the detailed mechanisms remain to be resolved.

It has been reported that *H. pylori* infection induces the expression of pro-inflammatory cytokines and enzymes such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in the gastric mucosa of rodents and humans (Jackson et al., 2000; Yamaoka et al., 2005; Bancel et al., 2006). In addition, a recent *in vitro* study demonstrated that NaCl could affect the production of interleukin (IL)-1ß, IL-6 and tumor necrosis factor-α induced by VacA, which is a virulence factor of *H. pylori*, in the AGS gastric cancer cell line (Sun et al., 2006). To our knowledge, however, there is limited information on the influence of long-term salt intake on *in vivo* expression of mediators of inflammation and proliferative activity. In the present study, we therefore examined whether a high-salt diet might increase epithelial proliferation and expression of iNOS and COX-2 assessed immunohistochemically in Mongolian gerbils at 40 weeks after *H. pylori* infection.

Materials and methods

Experimental design

The precise experimental design was as previously described (Kato et al., 2006). In the present study, 45 stomach samples from Mongolian gerbils (*Meriones unguiculatus*; MGS/Sea, Seac Yoshitomi, Fukuoka, Japan) were examined (Fig. 1). Briefly, the gerbils were divided into 4 groups (groups A-D). Groups A and B were inoculated with 1×10^8 colony-forming unit of *H. pylori* (ATCC43504, American Type Culture Collection, Rockville, MD, USA) intra-gastrically, while groups C and D were inoculated with sterile Brucella broth (Becton Dickinson, Cockeysville, MD, USA). *H. pylori* was prepared by the same method as described previously (Shimizu et al., 1999). From weeks 1 to 40, the animals of groups A and C received a diet including 10% sodium chloride and those in groups B and D were maintained on basal diet (CRF-1; Oriental Yeast Co. Ltd., Tokyo, Japan) containing 0.32% NaCl. At week 40, all gerbils were intraperitoneally injected with 5'-bromo-2'-deoxyuridine (BrdU) at a dose of 100 mg/kg, 60 minutes before sacrifice. The animals were subjected to deep anesthesia and laparotomy with excision of the stomach. The excised stomachs were fixed in 10% neutral-buffered formalin and sliced along the longitudinal axis into 4 to 8 strips of equal width, embedded in paraffin, and stained with hematoxylin and eosin (H&E) for histological examination. The experimental design was approved by the Animal Care Committee of Aichi Cancer Center Research Institute, and the animals were cared for in accordance with the institutional guidelines.

Immunohistochemistry to assess epithelial proliferation and inflammatory enzymes

Immunohistochemical analysis of BrdU, iNOS and COX-2 was carried out as previously described (Ikeno et al., 1999; Tanaka et al., 2006). Briefly, serial sections were deparaffinized and hydrated through a graded series of ethanols, and immersed in 0.3% hydrogen peroxide/methanol solution for inhibition of endogenous peroxidase activity. For antigen retrieval, sections for iNOS and COX-2 were microwaved in 10 mM citrate buffer (pH 6.0) for 10 minutes, and sections for BrdU were incubated in 5N hydrochloric acid for 30 minutes at room temperature. The sections were then incubated with primary antibodies: a mouse monoclonal anti-BrdU antibody (clone Bu20a, diluted 1:1,000, Dako, Glostrup, Denmark), a rabbit polyclonal anti-iNOS antibody (Saito et al., 2002) (diluted 1:500, Calbiochem, San Diego, CA, USA), or a mouse monoclonal anti-COX-2 antibody (Marrogi et al., 2000; Shiotani et al., 2001) (clone 33, diluted 1:100, BD Biosciences, San Jose, CA, USA). Staining for BrdU and COX-2 was performed using a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) and the Fast Red Substrate System (Dako), respectively. Sections for iNOS were incubated with biotinylated secondary antibody (swine-anti rabbit IgG, Dako), and avidin-biotinylated horseradish peroxidase complexes were visualized using 0.05% 3,3'-diaminobenzidine. All sections were counterstained with hematoxylin.

The numbers of BrdU-labeled cells in gastric mucosa were counted under a microscope, and indices were determined as the mean percentages of positive epithelial cells among totals of 90 different arbitrarily selected glands (including 60 glands in each corpus and 30 in each antrum). The degree of iNOS immuno-

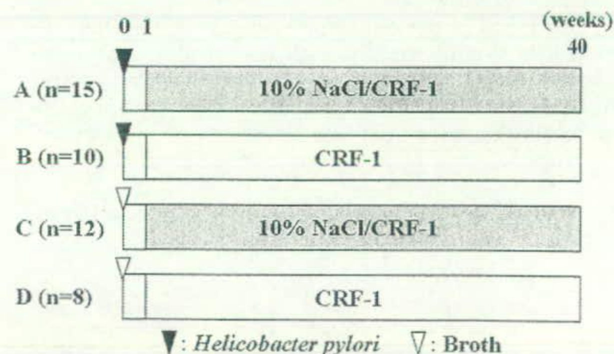

Fig. 1. Experimental design. Specific pathogen-free male, 4-month-old Mongolian gerbils were inoculated with *H. pylori* ATCC43504 strain (groups **A and B**) or Broth (groups **C and D**). Animals of groups A and C were given CRF-1 diet containing 10% NaCl from weeks 1 to 40.

positivity was expressed as the numbers of iNOS-positive cells in the total mucosal length. To quantitate the degree of COX-2 stainability, we measured the length of COX-2 positive areas per total mucosal length. The average mucosal lengths measured for evaluation of iNOS and COX-2 expression were 75.4±20.7 and 76.2±21.1 mm (means±SD), respectively.

Statistical analysis

Differences in data between groups were analyzed using the two-way factorial analysis of variance (ANOVA), followed by the Scheffe's multiple comparison procedure. P values <0.05 were considered to be statistically significant.

Results

Macroscopic and histological findings

The gastric mucosa of all gerbils in groups A and B (*H. pylori*-infected groups) was generally thickened and

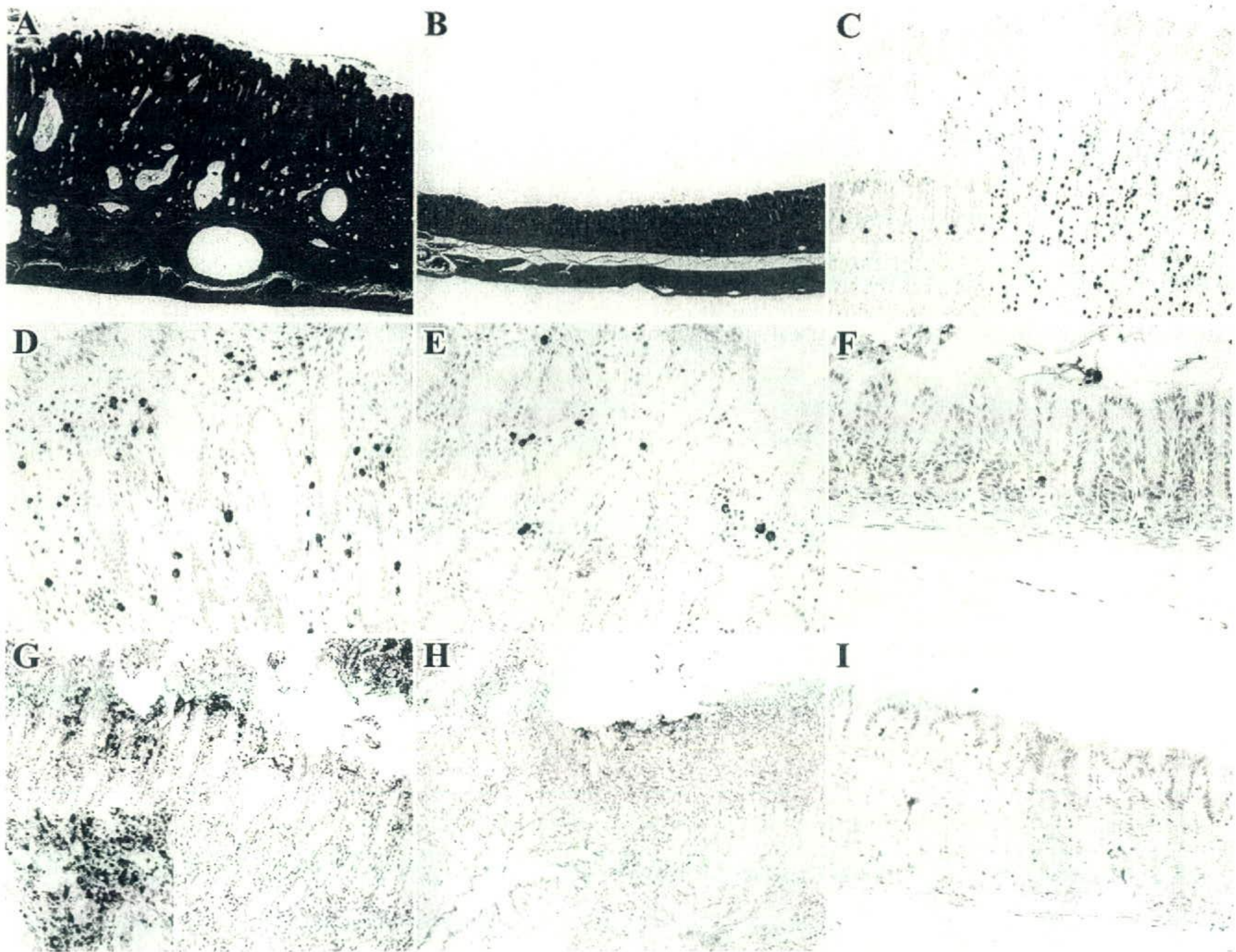

Fig. 2. Histopathology and immunohistochemistry of gastric mucosa of Mongolian gerbils. *H. pylori* + 10% NaCl group (**A, C, D and G**), *H. pylori* + basal diet group (**E and H**) and Broth + 10% NaCl group (**B, inset in C, F and I**). A and B. H&E staining. **A.** Note severe gastritis with infiltration of inflammatory cells, heterotopic proliferative glands, mucosal hyperplasia, and intestinal metaplasia at 40 weeks post-infection. x 50. **B.** No lesions were observed in gastric mucosa of non-infected and 10% NaCl diet-treated gerbils. x 50. **C.** Immunohistochemistry for BrdU. Large numbers of BrdU-positive cells are apparent in hyperplastic mucosal epithelium, while much fewer are present in the proliferative zone of a non-infected animal (inset). x 100. **D-F.** Immunohistochemistry for iNOS. **D and E.** Expression of iNOS mainly in mononuclear cells infiltrating in the lamina propria. x 200. **F.** In non-infected group, iNOS-positive cells were rarely observed in the lamina propria. x 200. **G-I.** Immunohistochemistry for COX-2. **G and H.** COX-2 is predominantly localized at the rims of areas of erosion or ulceration. Note expression localized in the cytoplasm of infiltrating mononuclear cells, fibroblasts and endothelium (**inset of G**). x 125. **I.** In the non-infected group, COX-2 staining was occasionally found in macrophages and endothelium. x 200.

edematous, occasionally with erosion and ulcers. In groups A and B, marked infiltration of neutrophils and mononuclear cells and formation of heterotopic proliferative glands were observed in the lamina propria and submucosa, occasionally with formation of lymphoid follicles. The histological examination also revealed various degrees of hyperplasia of the mucosa and intestinal metaplasia (Fig. 2A). Such macroscopic and histological lesions were not recognized in the stomachs of groups C and D (non-infected groups) (Fig. 2B). Detailed data for gastritis, including inflammation scores, have previously reported by our colleagues (Kato et al., 2006).

BrdU labeling indices for epithelial cells

BrdU-labeled epithelial cells in gastric mucosa were distributed mostly in the neck region of the hyperplastic polyps or in the proliferative zone of the non-hyperplastic mucosa (Fig. 2C). At week 40, BrdU labeling indices in *H. pylori*-infected groups were significantly greater than in non-infected groups ($P<0.0001$) (Fig. 3). The high-salt diet showed no significant effects of enhancement of epithelial proliferation both in *H. pylori*-infected groups and non-infected groups. There was no significant correlation between *H. pylori* infection and 10% NaCl diet in BrdU labeling indices ($P=0.4785$).

Immunohistochemistry of iNOS

In *H. pylori*-infected gerbils, immunostaining for iNOS was located mainly in the cytoplasm of infiltrating mononuclear cells both in the lamina propria and submucosa (Fig. 2D,E). Expression was also detected in endothelium, segmented leukocytes, and gastric epithelial cells at lower frequency. At 40 weeks, iNOS expression in group A (*H. pylori*-infected and 10% NaCl diet-treated group) was significantly higher than in

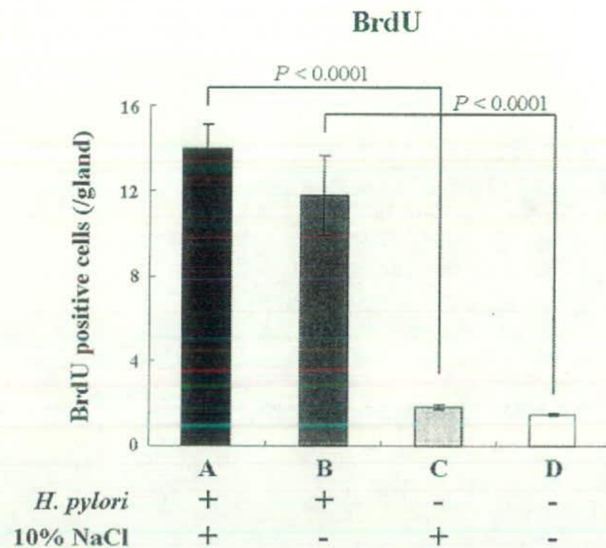

Fig. 3. Immunohistochemical analysis of epithelial cell proliferation in gastric mucosa of Mongolian gerbils by BrdU staining. Data are mean ± SE values.

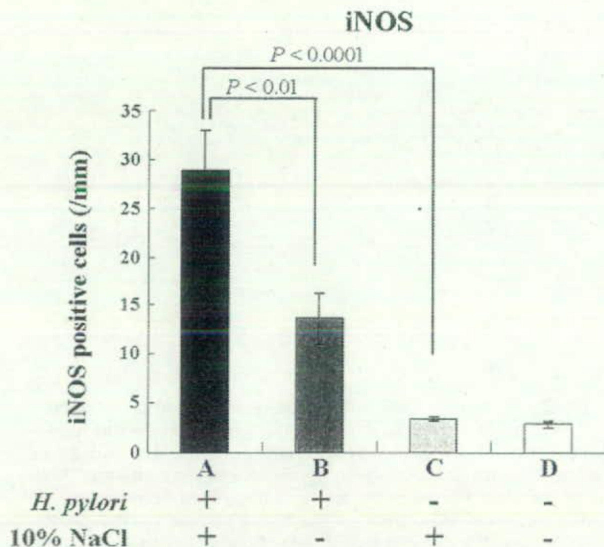

Fig. 4. Immunohistochemical analysis of iNOS expression in gastric mucosa of Mongolian gerbils. Data are mean ± SE values.

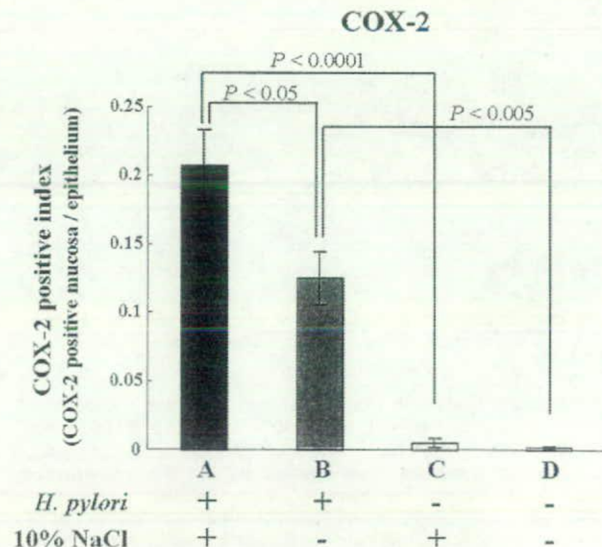

Fig. 5. Immunohistochemical analysis of COX-2 expression in gastric mucosa of Mongolian gerbils. Data are mean ± SE values.

group C (non-infected and 10% NaCl diet-treated group) ($P<0.0001$) (Fig. 4). Two-way factorial ANOVA revealed a significant interaction between *H. pylori* infection and excessive salt intake on iNOS expression ($P<0.05$). In *H. pylori*-infected groups, the numbers of iNOS-positive cells in group A (10% NaCl diet-treated) (28.8±4.12 cells/mm; means ± SE) were significantly higher than in group B (basal diet-treated) (13.7±2.63) ($P<0.01$). In non-infected groups, a high-salt diet showed no significant influence on frequency of iNOS expression (Fig. 2F).

Immunohistochemistry of COX-2

In *H. pylori*-infected groups, COX-2 protein was mainly detected in infiltrating mononuclear cells, fibroblasts, and endothelium in the lamina propria, particularly at the rims of erosion and ulcers (Fig. 2G,H), while a little COX-2 staining was observed in mononuclear cells and endothelium of non-infected gerbils (Fig. 2I). At 40 weeks, COX-2 expression in groups A and B (*H. pylori*-infected) was significantly greater than in groups C and D (non-infected) ($P<0.0001$ and $P<0.005$, respectively) (Fig. 5). Two-way factorial ANOVA showed a tendency for interaction between *H. pylori* infection and 10% NaCl diet on COX-2 expression, although this was not statistically significant ($P=0.0599$). In *H. pylori*-infected groups, the COX-2 positive index in group A (10% NaCl diet-treated) (0.21±0.03; means ± SE) was significantly higher than that in group B (basal diet-treated) (0.12±0.02) ($P<0.05$). There were no significant effects of salt on COX-2 immunoreactivity between the non-infected groups.

Discussion

It has been recognized that iNOS and COX-2 are involved in the processes of inflammation, carcinogenesis and its progression (Prescott and Fitzpatrick, 2000; Jaiswal et al., 2001). iNOS is expressed both by inflammatory cells and epithelial cells and the generated nitric oxide contributes to carcinogenesis during chronic inflammation. COX-2 is an inducible form of cyclooxygenase, which catalyzes the conversion of arachidonic acid to pro-carcinogenic eicosanoids such as prostaglandin, and is increased by various cytokines, growth factors and reactive oxygen species. A number of previous findings suggest that both iNOS and COX-2 are associated with *H. pylori*-induced gastritis in humans (Mannick et al., 1996; Jackson et al., 2000; Bhandari et al., 2005). In the present study, we showed COX-2 expression in gastric mucosa to be significantly enhanced by *H. pylori* infection in Mongolian gerbils, consistent with previous immunohistochemical studies in humans (Fu et al., 1999; Chen et al., 2006). In addition, our results demonstrated that a high-salt diet can further upregulate the expression of these two enzymes in *H. pylori*-infected gerbils. To our knowledge, this is the first report of synergistic effects of salt and *H. pylori* infection on the expression of iNOS and COX-2 in the glandular stomach of Mongolian gerbils. Rajnakova et al. (2001) reported using immunohistochemistry that iNOS and COX-2 expression may promote gastric cancer progression associated with an accumulation of p53. Furthermore, prognosis in patients expressing both iNOS and COX-2 appears to be significantly poorer than in those with single or no expression of these two genes (Chen et al., 2006). The results thus indicate a possibility that the co-expression of iNOS and COX-2 may not only promote gastric inflammation but also be a determinant factor for *H. pylori*-associated gastric carcinogenesis and prognostic outcome.

We have previously reported that excessive salt intake enhances *H. pylori*-associated gastritis and gastric cancer development in gerbils through alteration of the gastric mucus microenvironment (Kato et al., 2006). The present study showed that chronic salt administration enhances iNOS and COX-2 expression in gastric mucosa of *H. pylori*-infected gerbils. In non-infected gerbils, on the other hand, salt alone induced almost no lesions in stomach mucosa and had no promoting effects on iNOS and COX-2 expression. Furthermore, we found a significant synergistic effect between excessive salt intake and *H. pylori* infection on iNOS expression and a tendency to enhance COX-2 expression. The results suggest that increased expression of iNOS and COX-2 was induced not by a high-salt diet alone but by promoting effects of salt on *H. pylori*-activated inflammatory responses, and that excessive salt intake may be associated with the progression of *H. pylori*-induced gastritis. Further analysis is needed to clarify the interaction between co-expression of iNOS and COX-2 and progression of gastritis, because over-expression is not directly linked to functional activation.

In the present study, COX-2 immunostaining was observed in infiltrated mononuclear cells, fibroblasts and endothelium in the lamina propria, particularly at the edges of erosions and ulcers, consistent with previous reports on ulcerated gastric mucosa in humans and rodents (Mizuno et al., 1997; Takahashi et al., 1998; Jackson et al., 2000). These studies suggested that the localization of COX-2 may be associated with repair of mucosal injury. Our present results showed that excessive salt intake could significantly increase COX-2 expression in *H. pylori*-infected gerbils, without any influence on the localization. Since salt alone had no significant effects on COX-2 expression in non-infected gerbils, further up-regulation of COX-2 by salt intake in *H. pylori*-infected gerbils may be related to enhancement of *H. pylori*-induced gastritis rather than direct mucosal damage.

Several studies in rats have demonstrated that acute exposure to a highly concentrated NaCl solution may cause direct injury of the gastric surface epithelium, followed by rapid recovery with increased regenerative cellular proliferation (Charnley and Tannenbaum, 1985; Furihata et al., 1996). In the present study, on the other

hand, a 10% NaCl-containing diet had no significant effects on epithelial proliferation in gastric mucosa, independent of *H. pylori* infection. In addition, our previous study demonstrated that intermittent (once a week) administration of saturated NaCl solution for 40 weeks had no promoting effects on *H. pylori*-associated gastritis and carcinogenesis in gerbils (Kato et al., 2006). Therefore, we consider that continuous exposure to salt, rather than short-term and highly-concentrated salt intake, may be important to enhance *H. pylori*-induced gastritis, associated with increased expression of iNOS and COX-2 in the gastric mucosa. A recent study reported that osmoprotective genes promote cell survival against NaCl-induced hypertonic stress (Neuhofer et al., 2007). The osmoprotective activity might be one of the determinant factors for outcome with gastric epithelial cells exposed to various concentrations of NaCl, although detailed functions in the stomach are little understood.

In conclusion, the present study showed synergistic effects of salt with *H. pylori* infection on iNOS and COX-2 expression in the gastric mucosa of Mongolian gerbils. The results provide further support for the hypothesis that salt promotes progression of *H. pylori*-induced gastritis, and also raise the possibility that reduction of salt intake may decrease the risk of *H. pylori*-associated gastric cancer, compatible with previous epidemiological and experimental findings.

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References

Bancel B., Esteve J., Souquet J.C., Toyokuni S., Ohshima H. and Pignatelli B. (2006). Differences in oxidative stress dependence between gastric adenocarcinoma subtypes. World J. Gastroenterol. 12, 1005-1012.

Bhandari P., Bateman A.C., Mehta R.L. and Patel P. (2005). Mucosal expression of cyclooxygenase isoforms 1 and 2 is increased with worsening damage to the gastric mucosa. Histopathology 46, 280-286.

Cao X., Tsukamoto T., Nozaki K., Tanaka H., Cao L., Toyoda T., Takasu S., Ban H., Kumagai T. and Tatematsu M. (2007). Severity of gastritis determines glandular stomach carcinogenesis in Helicobacter pylori-infected Mongolian gerbils. Cancer Sci. 98, 478-483.

Charnley G. and Tannenbaum S.R. (1985). Flow cytometric analysis of the effect of sodium chloride on gastric cancer risk in the rat. Cancer Res. 45, 5608-5616.

Chen C.N., Hsieh F.J., Cheng Y.M., Chang K.J. and Lee P.H. (2006). Expression of inducible nitric oxide synthase and cyclooxygenase-2 in angiogenesis and clinical outcome of human gastric cancer. J. Surg. Oncol. 94, 226-233.

Correa P. (1995). Helicobacter pylori and gastric carcinogenesis. Am. J. Surg. Pathol. 19 (Suppl 1), S37-43.

Coussens L.M. and Werb Z. (2002). Inflammation and cancer. Nature 420, 860-867.

Fox J.G., Dangler C.A., Taylor N.S., King A., Koh T.J. and Wang T.C. (1999). High-salt diet induces gastric epithelial hyperplasia and parietal cell loss, and enhances Helicobacter pylori colonization in C57BL/6 mice. Cancer Res. 59, 4823-4828.

Fu S., Ramanujam K.S., Wong A., Fantry G.T., Drachenberg C.B., James S.P., Meltzer S.J. and Wilson K.T. (1999). Increased expression and cellular localization of inducible nitric oxide synthase and cyclooxygenase 2 in *Helicobacter pylori* gastritis. Gastroenterology 116, 1319-1329.

Furihata C., Ohta H. and Katsuyama T. (1996). Cause and effect between concentration-dependent tissue damage and temporary cell proliferation in rat stomach mucosa by NaCl, a stomach tumor promoter. Carcinogenesis 17, 401-406.

Gamboa-Dominguez A., Ubbelohde T., Saqui-Salces M., Romano-Mazzoti L., Cervantes M., Dominguez-Fonseca C., de la Luz Estreber M. and Ruiz-Palacios G.M. (2007). Salt and Stress Synergize H. pylori-Induced Gastric Lesions, Cell Proliferation, and p21 Expression in Mongolian Gerbils. Dig. Dis. Sci. 52, 1517-1526

Hirayama F., Takagi S., Yokoyama Y., Iwao E. and Ikeda Y. (1996). Establishment of gastric Helicobacter pylori infection in Mongolian gerbils. J. Gastroenterol. 31 Suppl 9, 24-28.

IARC Working Group on the Evaluation of Carcinogenic Risks to Humans (1994) Infection with *Helicobacter pylori*. In: Schistosomes, Liver Flukes and *Helicobacter pylori*, IARC Monographs on the Evalutaion of Carcinogenic Risks to Humans. Vol. 61. World Health Organization / International Agency for Research on Cancer, Lyon. pp 177-241.

Ikeno T., Ota H., Sugiyama A., Ishida K., Katsuyama T., Genta R.M. and Kawasaki S. (1999). Helicobacter pylori-induced chronic active gastritis, intestinal metaplasia, and gastric ulcer in Mongolian gerbils. Am. J. Pathol. 154, 951-960.

Jackson L.M., Wu K.C., Mahida Y.R., Jenkins D. and Hawkey C.J. (2000). Cyclooxygenase (COX) 1 and 2 in normal, inflamed, and ulcerated human gastric mucosa. Gut 47, 762-770.

Jaiswal M., LaRusso N.F. and Gores G.J. (2001). Nitric oxide in gastrointestinal epithelial cell carcinogenesis: linking inflammation to oncogenesis. Am. J. Physiol. Gastrointest. Liver Physiol. 281, G626-634.

Joossens J.V., Hill M.J., Elliott P., Stamler R., Lesaffre E., Dyer A., Nichols R. and Kesteloot H. (1996). Dietary salt, nitrate and stomach cancer mortality in 24 countries. European Cancer Prevention (ECP) and the INTERSALT Cooperative Research Group. Int. J. Epidemiol. 25, 494-504.

Kato S., Tsukamoto T., Mizoshita T., Tanaka H., Kumagai T., Ota H., Katsuyama T., Asaka M. and Tatematsu M. (2006). High salt diets dose-dependently promote gastric chemical carcinogenesis in Helicobacter pylori-infected Mongolian gerbils associated with a shift in mucin production from glandular to surface mucous cells. Int. J. Cancer 119, 1558-1566.

Kono S. and Hirohata T. (1996). Nutrition and stomach cancer. Cancer Causes Control 7, 41-55.

Mannick E.E., Bravo L.E., Zarama G., Realpe J.L., Zhang X.J., Ruiz B., Fontham E.T., Mera R., Miller M.J. and Correa P. (1996). Inducible nitric oxide synthase, nitrotyrosine, and apoptosis in Helicobacter pylori gastritis: effect of antibiotics and antioxidants. Cancer Res. 56, 3238-3243.

Marrogi A., Pass H.I., Khan M., Metheny-Barlow L.J., Harris C.C. and Gerwin B.I. (2000). Human mesothelioma samples overexpress both cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (NOS2): in vitro antiproliferative effects of a COX-2 inhibitor. Cancer Res. 60, 3696-3700.

Marshall B.J. and Warren J.R. (1984). Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. Lancet 1, 1311-1315.

Mizuno H., Sakamoto C., Matsuda K., Wada K., Uchida T., Noguchi H., Akamatsu T. and Kasuga M. (1997). Induction of cyclooxygenase 2 in gastric mucosal lesions and its inhibition by the specific antagonist delays healing in mice. Gastroenterology 112, 387-397.

Neuhofer W., Steinert D., Fraek M.L. and Beck F.X. (2007). Prostaglandin E2 stimulates expression of osmoprotective genes in MDCK cells and promotes survival under hypertonic conditions. J. Physiol. 583, 287-297.

Prescott S.M. and Fitzpatrick F.A. (2000). Cyclooxygenase-2 and carcinogenesis. Biochim. Biophys. Acta 1470, M69-78.

Rajnakova A., Moochhala S., Goh P.M. and Ngoi S. (2001). Expression of nitric oxide synthase, cyclooxygenase, and p53 in different stages of human gastric cancer. Cancer Lett. 172, 177-185.

Saito T., Hu F., Tayara L., Fahas L., Shennib H. and Giaid A. (2002). Inhibition of NOS II prevents cardiac dysfunction in myocardial infarction and congestive heart failure. Am. J. Physiol. Heart Circ. Physiol. 283, H339-345.

Shikata K., Kiyohara Y., Kubo M., Yonemoto K., Ninomiya T., Shirota T., Tanizaki Y., Doi Y., Tanaka K., Oishi Y., Matsumoto T. and Iida M. (2006). A prospective study of dietary salt intake and gastric cancer incidence in a defined Japanese population: the Hisayama study. Int. J. Cancer 119, 196-201.

Shimizu N., Inada K.I., Tsukamoto T., Nakanishi H., Ikehara Y., Yoshikawa A., Kaminishi M., Kuramoto S. and Tatematsu M. (1999). New animal model of glandular stomach carcinogenesis in Mongolian gerbils infected with *Helicobacter pylori* and treated with a chemical carcinogen. J. Gastroenterol. 34 (Suppl. 11), 61-66.

Shiotani H., Denda A., Yamamoto K., Kitayama W., Endoh T., Sasaki Y., Tsutsumi N., Sugimura M. and Konishi Y. (2001). Increased expression of cyclooxygenase-2 protein in 4-nitroquinoline-1-oxide-induced rat tongue carcinomas and chemopreventive efficacy of a specific inhibitor, nimesulide. Cancer Res. 61, 1451-1456.

Sugiyama A., Maruta F., Ikeno T., Ishida K., Kawasaki S., Katsuyama T., Shimizu N. and Tatematsu M. (1998). Helicobacter pylori infection enhances N-methyl-N-nitrosourea-induced stomach carcinogenesis in the Mongolian gerbil. Cancer Res. 58, 2067-2069.

Sun J., Aoki K., Zheng J.X., Su B.Z., Ouyang X.H. and Misumi J. (2006). Effect of NaCl and Helicobacter pylori vacuolating cytotoxin on cytokine expression and viability. World J. Gastroenterol. 12, 2174-2180.

Tajima K. and Tominaga S. (1985). Dietary habits and gastro-intestinal cancers: a comparative case-control study of stomach and large intestinal cancers in Nagoya, Japan. Jpn. J. Cancer Res. 76, 705-716.

Takahashi S., Shigeta J., Inoue H., Tanabe T. and Okabe S. (1998). Localization of cyclooxygenase-2 and regulation of its mRNA expression in gastric ulcers in rats. Am. J. Physiol. 275, G1137-1145.

Tanaka T., Kohno H., Suzuki R., Hata K., Sugie S., Niho N., Sakano K., Takahashi M. and Wakabayashi K. (2006). Dextran sodium sulfate strongly promotes colorectal carcinogenesis in Apc(Min/+) mice: inflammatory stimuli by dextran sodium sulfate results in development of multiple colonic neoplasms. Int. J. Cancer 118, 25-34.

Tsugane S. (2005). Salt, salted food intake, and risk of gastric cancer: epidemiologic evidence. Cancer Sci. 96, 1-6.

Tsugane S., Sasazuki S., Kobayashi M. and Sasaki S. (2004). Salt and salted food intake and subsequent risk of gastric cancer among middle-aged Japanese men and women. Br. J. Cancer 90, 128-134.

Uemura N., Okamoto S., Yamamoto S., Matsumura N., Yamaguchi S., Yamakido M., Taniyama K., Sasaki N. and Schlemper R.J. (2001). Helicobacter pylori infection and the development of gastric cancer. N. Engl. J. Med. 345, 784-789.

Yamaoka Y., Yamauchi K., Ota H., Sugiyama A., Ishizone S., Graham D.Y., Maruta F., Murakami M. and Katsuyama T. (2005). Natural history of gastric mucosal cytokine expression in *Helicobacter pylori* gastritis in Mongolian gerbils. Infect. Immun. 73, 2205-2212.

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Roles of cyclooxygenase-2 and microsomal prostaglandin E synthase-1 expression and β-catenin activation in gastric carcinogenesis in *N*-methyl-*N*-nitrosourea-treated K19-C2mE transgenic mice

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K19-C2mE transgenic (Tg) mice, simultaneously expressing cyclooxygenase-2 (COX-2) and microsomal prostaglandin E synthase-1 (mPGES-1) in the gastric mucosa under the cytokeratin 19 gene promoter, were here treated with *N*-methyl-*N*-nitrosourea (MNU) and inoculated with *Helicobacter pylori* (*H. pylori*) to investigate gastric carcinogenesis. Wild-type (WT) and Tg mice undergoing MNU treatment frequently developed tumors in the pyloric region (100% and 94.7%, respectively); multiplicity in Tg was higher than that in WT ($P < 0.05$) with *H. pylori* infection. Larger pyloric tumors were more frequently observed in Tg than in WT ($P < 0.05$). In addition, Tg developed fundic tumors, where WT did not. No gastric tumors were observed without MNU treatment. Transcripts of TNF-α, iNOS, IL-1β, and CXCL14 were up-regulated with *H. pylori* infection in both genotypes and were also increased more in Tg than in WT within *H. pylori*-inoculated animals. Immunohistochemical analysis demonstrated significantly greater β-catenin accumulation in pyloric tumors, compared with those in the fundus ($P < 0.01$) with mutations of exon 3; 18.2% and 31.6% in MNU-alone and MNU + *H. pylori*-treated WT, whereas 21.4% and 62.5% was observed in the Tg, respectively; the latter significantly higher ($P < 0.05$), suggesting the role of *H. pylori* in Wnt activation. In conclusion, K19-C2mE mice promoted gastric cancer in both fundic and pyloric regions. Furthermore β-catenin activation may play the important role of pyloric carcinogenesis especially in *H. pylori*-infected Tg. Induction of various inflammatory cytokines in addition to overexpression of COX-2/mPGES-1 could be risk factors of gastric carcinogenesis and may serve as a better gastric carcinogenesis model. (*Cancer Sci* 2008; 99: 2356–2364)

There is a large body of evidence that *Helicobacter pylori* (*H. pylori*) infection is involved in development of chronic gastritis, peptic ulceration, and gastric cancer.[1,2] Recent reports have revealed that *H. pylori* infection induces cyclooxygenase-2 (COX-2) expression and microsomal prostaglandin E synthase-1 (mPGES-1), enzymes responsible for synthesizing prostaglandin E_2 (PGE_2) in gastric mucosa.[3–5] In the stomach, prostaglandins are major molecules for maintaining the gastric mucosa.[6] PGE_2 plays distinct roles in tumor growth and metastasis in several cancers.[7]

Oshima *et al.* recently constructed transgenic mice (K19-C2mE) that simultaneously overexpress COX-2 and mPGES-1 in the gastric mucosa under the influence of the cytokeratin 19 gene promoter.[4] The transgenic (Tg) mice develop inflammation-associated hyperplastic lesions in the proximal glandular stomach, similar to those found in the *Helicobacter*-infected stomach.[4] Furthermore, K19-Wnt1/C2mE Tg mice, simultaneously expressing Wnt1 as well as COX-2/mPGES-1, develop dysplastic gastric tumors,[8] indicating that COX-2/mPGES-1 and Wnt pathway activation might be involved in mouse gastric neoplasia.

The Wnt/β-catenin signaling pathway plays important roles in cell–cell adhesion and cell cycle regulation and its alternation is implicated in genesis of many cancers. Abnormal nuclear accumulation of β-catenin due to mutation of the β-catenin gene stimulates the expression of β-catenin/Tcf target genes, such as *c-myc*, *c-jun*, and *cyclin D1*.[9,10] Mutations of β-catenin gene exon 3, where serine and threonine residues are physiologically phosphorylated by glycogen synthase kinase (GSK)-3β, prevent degeneration by APC/GSK-3β/Axin complex. With human gastric cancers, nuclear accumulation of β-catenin has been estimated to occur in 12–37% of cases,[11–15] with mutations in exon 3 reported in a few to over 20%.[12,16,17] However, the degree of involvement of Wnt pathway alteration in the development of mouse gastric cancers remains unclear.

In the present study, Tg mice were treated with a stomach carcinogen, *N*-methyl-*N*-nitrosourea (MNU), and inoculated with *H. pylori* to investigate the influence of COX-2/mPGES-1 expression and *H. pylori* infection on mouse gastric carcinogenesis. Furthermore, we analyzed the frequency of β-catenin activation and gene mutations to assess involvement of the Wnt pathway.

Materials and Methods

Experimental design. The experimental design is shown in Fig. 1. K19-C2mE Tg mice and littermate wild-type (WT) mice were randomly divided into four groups (groups A–D). The mice of groups B and D were inoculated intragastrically with 0.8 mL of broth culture containing *H. pylori*. After 1 week, the mice of groups C and D were given MNU (Sigma Chemical Co., St Louis, MO, USA) in drinking water at the concentration of 120 p.p.m. on alternate weeks (total exposure was 5 weeks) and then normal tap water until the end of experiment. MNU was dissolved in distilled water and freshly prepared three times per week. At the end of the experiment, all surviving mice were sacrificed under deep anesthesia 60 min after an intraperitoneal

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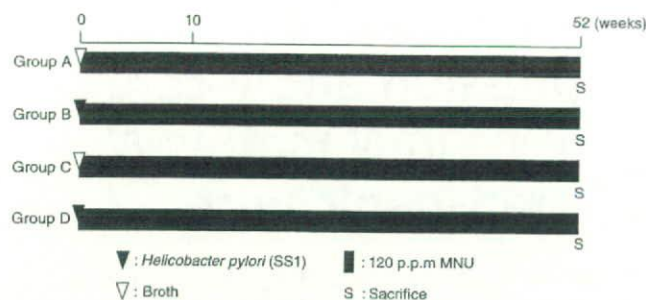

Fig. 1. Experimental design. Five- to 6-week-old K19-C2mE transgenic mice and littermate wild-type mice were inoculated with *Helicobacter pylori* SS1 (groups B and D) or broth (groups A and C). After 1 week, animals of groups C and D were administered 120 p.p.m. *N*-methyl-*N*-nitrosourea on alternate weeks (total exposure, 5 weeks).

injection of 5′-bromo-2′-deoxyuridine (BrdU) at a dose of 100 mg/kg. The excised stomachs were fixed in 10% neutral-buffered formalin or 95% ethanol plus 1% acetic acid for histology and immunohistochemistry.

Animals. K19-C2mE transgenic (Tg) mice produced by Oshima *et al.*[4] were maintained by breeding male K19-C2mE Tg with female C57BL/6 N at the Animal Facility of Aichi Cancer Center Research Institute. WT mice were used as controls. All were housed in plastic cages with hardwood chips in an air-conditioned room with 12 h light–12 h dark cycle and given a basal diet (CA-1; CLEA Japan Inc., Tokyo, Japan) and water *ad libitum*. For genotyping of each mouse, DNA samples were extracted from the tails using a DNeasy tissue kit (Qiagen, Tokyo, Japan) and subjected to polymerase chain reaction (PCR) as reported elsewhere.[4]

Bacterial culture. *H. pylori* strain SS1 was inoculated on Brucella agar plates (Merck, Darmstadt, Germany) containing 7% v/v heat-inactivated fetal calf serum and incubated at 37°C under microaerobic conditions using an Anaero Pack Campylo (Mitsubishi Gas Chemical Co., Tokyo, Japan) at high humidity for 2 days. Then, bacteria grown on the plates were introduced into Brucella broth (Becton Dickson, Cockeysville, MD, USA) supplemented with 7% v/v fetal calf serum, and cultures of *H. pylori* were checked under a phase contrast microscope for bacterial shape and mobility.

Histopathological analysis. Tissue sections were stained with hematoxylin–eosin and Alcian blue (pH 2.5)–periodic acid-Schiff (AB-PAS) for histological analysis. Tumor location was categorized into 'fundic' and 'pyloric', the former included tumors developing in the gastric fundic mucosa and border areas of fundic and pyloric glands. The glandular mucosa was examined histologically for any inflammatory and epithelial changes. Active chronic gastritis was estimated according to criteria modified from the updated Sydney System, characterized by infiltration of neutrophils and lymphocytes. The degree of change was graded in a scale from 0 to 3, (0 [normal], 1 [mild], 2 [moderate], and 3 [marked]). Mucosa thickness was measured using AxioVision 4.6 (Carl Zeiss, Jena, Germany). Tumor size was evaluated by the largest tumor area using NIH image version 1.62 (National Institutes of Health, USA) on hematoxylin–eosin sections. Serial sections were also stained immunohistochemically with antibodies against COX-2 (Cayman Chemical, Ann Arbor, MI, USA), β-catenin (clone 14; BD Transduction Laboratories, KY, USA), and BrdU (Dako, Glostrup, Denmark). BrdU labeling index was calculated as the percentages of BrdU-positive epithelial cells within glands at five different arbitrarily selected points in gastric mucosa.

Analysis of inflammatory cytokine mRNA by quantitative real-time reverse transcription (RT)–PCR. Total RNA was extracted from the

Table 1. Primer sequences used for quantitative reverse transcription–polymerase chain reaction

Target	Primer sequence	Product size (bp)
COX-2	5′-AAGCCCTCTACAGTGACATC-3′ 5′-GAGAATGGTGCTCCAAGCTCTA-3′	115
TNF-α	5′-GCCGATGGGTTGTACCTTGTCTACT-3′ 5′-ACGGCAGAGAGGAGGTTGACTT-3′	134
iNOS	5′-CCGGCAAACCCAAGGTCTACGTT-3′ 5′-CACATCCCGAGCCATGCGCACATCT-3′	128
IL-1β	5′-TTGACTTCACCATGGAATCCGTGTC-3′ 5′-GAGTCCCCTGGAGATTGAGC-3′	126
IL-6	5′-CCTACCCCAATTTCCAATGCTCT-3′ 5′-CACTAGGTTTGCCGAGTAGATCTCA-3′	143
CXCL14	5′-TGGTTGAGACCGTTCACAGCACTAC-3′ 5′-GAAACTCTGACCAGTCATAAGCC-3′	122
GAPDH	5′-CAACTCCCACTCTTCCACCTTCGAT-3′ 5′-CCTGTTGCTGTAGCCGTATTC-3′	106

COX-2, cyclooxygenase-2; GAPDH, glyceraldhyde-3-phosphate dehydrogenase; IL-1β, interleukin-1β; IL-6, interleukin-6; iNOS, inducible nitric oxide synthase; TNF-α, tumor necrosis factor-α.

border areas of fundic and pyloric regions in the glandular stomach mucosa using an RNeasy Plus Mini kit (Qiagen, Hilden, Germany). First strand cDNAs were synthesized using a Super Script III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Relative quantitative PCR for COX-2, tumor necrosis factor-α (TNF-α), inducible nitric oxide synthase (iNOS), interleukin-1β (IL-1β), IL-6, and CXCL14 was performed using the mouse glyceraldhyde-3-phosphate dehydrogenase (GAPDH) gene as an internal control with the StepOne Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) using a QuantiTect SYBR Green PCR kit (Qiagen). The primer sequences are listed in Table 1. Quantification was performed as earlier established.[18] The expression levels were expressed relative to 1.00 in WT mice in the control group A.

DNA extraction and direct sequencing. Immunoreactivity of β-catenin was classified into 'nuclear/cytoplasmic' or 'membranous' according to the intracellular localization of β-catenin protein. Tumors with 5% or more section area of nuclear/cytoplasmic β-catenin were judged as β-catenin accumulating. Tumor areas with nuclear/cytoplasmic or membranous β-catenin localization and surrounding gastric mucosa in serial paraffin sections (5-μm thick) were microdissected using a laser microdissection system (AS LMD; Leica Microsystems, Wetzlar, Germany). Microdissection, PCR, and sequencing were performed as previously reported.[19,20] The PCR primer sequences to amplify exon 3 of mouse β-catenin gene were 5′-AGCCACTGGCAGCAGCAGTCTTAC-3′ and 5′-ATAAAGGACTTGGGAGGTGTCAACA-3′. Sequencing was performed using a BigDye Terminator Cycle Sequencing Kit (v 3.1; Applied Biosystems) with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

Statistical analysis. The incidences of gastric tumors and frequencies of β-catenin accumulation and gene mutation were analyzed using Fisher's exact probability test. Differences of inflammation scores were assessed with the Mann–Whitney *U*-test.

Results

Incidence, multiplicity, and size of gastric tumors. The observed incidences and multiplicities of gastric tumors are summarized in Table 2. In the fundic region, Tg mice of groups C and D developed dysplastic gastric tumors (Fig. 2Aa,b), whereas WT mice did not. In contrast, WT (Fig. 2Ba,b) and Tg (Fig. 2Ca,b)

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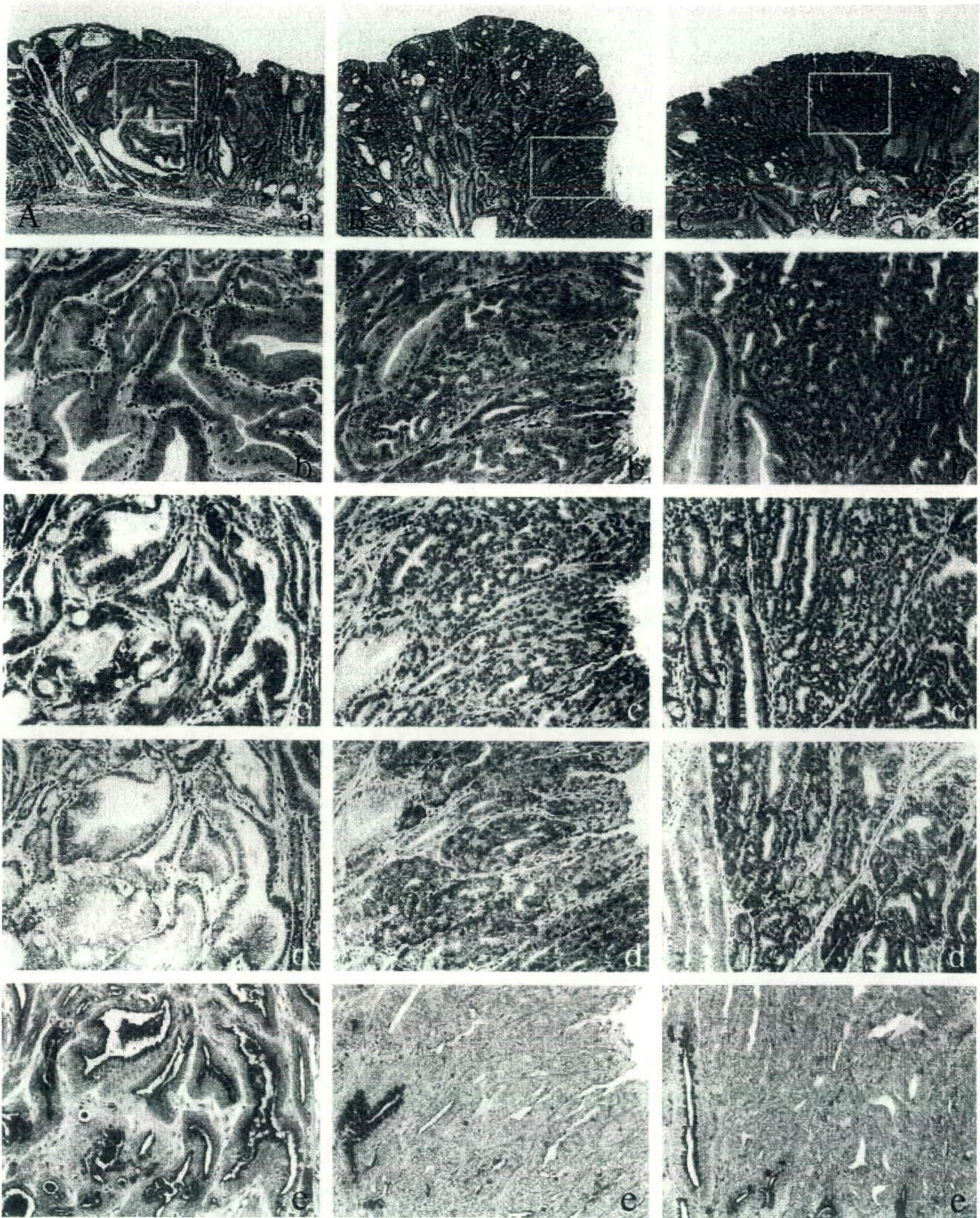

Fig. 2. Histopathological and immunohistochemical analysis of COX-2 and β-catenin in *N*-methyl-*N*-nitrosourea (MNU)–induced tumors in K19-C2mE Tg mice. (A) Gastric tumors in the fundic region of MNU-treated Tg mice. (B,C) Gastric tumors with nuclear β-catenin accumulation in the pyloric region of MNU-induced WT (B) and Tg mice (C). (a,b) Hematoxylin–eosin staining. (c,d) Immunohistochemistry for COX-2 (c) and β-catenin (d). (e) Alcian blue (pH 2.5)–periodic acid-Schiff (AB-PAS). Yellow arrow shows PAS-positive mucin. Yellow boxes in (a) are magnified in (b–e), respectively. Original magnification, 50× (a), 200× (b–e).

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Table 2. Incidence and multiplicity of MNU-treated K19-C2mE mice with gastric tumors

Groups	Treatments	Genotypes	Effective nos.	Incidence (%)		Tumor multiplicity in pyloric mucosa (no. of tumors/mouse)†
				Fundic mucosa	Pyloric mucosa	
A	Broth	WT	10	0 (0%)	0 (0%)	0
		Tg	10	0 (0%)	0 (0%)	0
B	*H. pylori*	WT	10	0 (0%)	0 (0%)	0
		Tg	10	0 (0%)	0 (0%)	0
C	Broth + MNU	WT	12	0 (0%)	12 (100%)	1.40 ± 0.70
		Tg	15	4 (26.7%)	15 (100%)	2.00 ± 0.88
D	*H. pylori* + MNU	WT	24	0 (0%)	24 (100%)	1.75 ± 0.74
		Tg	19	4 (21.1%)*	18 (94.7%)	2.28 ± 0.96*

H. pylori, *Helicobacter pylori*; MNU, *N*-methyl-*N*-nitrosourea; Tg, Transgenic; WT, wild type.
*$P < 0.05$ *versus* WT within group D.
†Values are expressed as average ± SD.

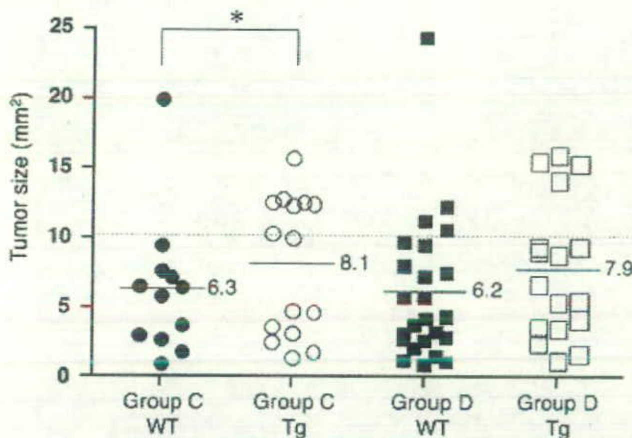

Fig. 3. Maximum tumor size in individual *N*-methyl-*N*-nitrosourea (MNU)–induced K19-C2mE mice in the pyloric region. Ratio of larger tumors (> 10.0 mm²): Tg *versus* WT in group C, 8/15 = 53.3% *versus* 1/12 = 8.3% (*$P < 0.05$); 4/24 = 16.7% *versus* 4/17 = 23.5% (not significant) in group D. Dotted line, cut-off value (10 mm²). Horizontal lines and values, mean.

mice of groups C and D developed dysplastic gastric tumors in the pyloric region; incidences were 100% and 94.7%, respectively, the difference not being significant among the groups and genotypes. Tumor multiplicity in Tg mice in group D (2.28 ± 0.96 tumors/mouse) was higher than those in WT mice in group C (1.40 ± 0.70) and in group D (1.75 ± 0.74) ($P < 0.01$ and $P < 0.05$, respectively). The maximum tumor size in each animal is plotted in Fig. 3. Averages ± SD were 6.3 ± 5.0 and 8.1 ± 5.0 in WT and Tg mice in group C. The corresponding figures were 6.2 ± 5.2 and 7.9 ± 5.1 in group D. The ratio of larger tumors (> 10.0 mm²) in Tg mice (8/15 = 53.3%) was significantly more frequent than that in the WT (1/12 = 8.3%) mice within group C ($P < 0.05$), but not within group D (4/24 = 16.7% and 4/17 = 23.5% in WT and Tg, respectively, $P = 0.70$). No gastric tumors were observed in groups A and B.

Status of gastritis. Data for the gastritis status in each group are summarized in Table 3. The gastric mucosa of *H. pylori* infected groups (B and D) showed significantly higher scores for infiltration of neutrophils and lymphocytes than the non-*H. pylori*-infected groups (A and C). There were no significant differences in scores for infiltration of neutrophils and lymphocytes between genotypes. Gastric mucosa of Tg mice was significantly thickened compared with that of WT. Correspondingly, gastric mucosa of mice with *H. pylori* infection was also significantly thickened compared with mice without *H. pylori* infection. BrdU labeling indices were increased with *H. pylori* infection irrespective of genotypes (groups B and D), associated with hyperplastic change in fundic mucosa. In pyloric mucosa, the BrdU labeling index was higher in Tg mice in group D.

Alteration of expression of inflammatory cytokines. Data for the expression levels of inflammatory factors in each group are summarized in Table 4. The mRNA expressions of TNF-α, iNOS, IL-1β, and CXCL14 in *H. pylori*-infected groups were significantly increased compared with non-*H. pylori*-infected groups in both genotypes. COX-2 was not up-regulated with *H. pylori* infection in WT animals. Furthermore, the mRNA expression levels of COX-2, TNF-α, iNOS, IL-1β, and CXCL14 in Tg mice were significantly higher than those in WT mice in group D. In the groups A–C, there were no significant differences in expression of inflammatory factors between both genotypes except for COX-2.

Immunolocalization of COX-2 and β-catenin, and mucin staining. COX-2 expression was retained in the fundic tumor cells (Fig. 2Ac) as well as surrounding normal foveolar epithelium (Fig. 2Cc, left image). But it was attenuated in pyloric tumor cells in WT (Fig. 2Bc) and Tg (Fig. 2Cc, right image) mice. Besides the epithelial tumor cells, infiltrating and/or stromal cells also expressed COX-2 in tumor stroma (right surface area in Fig. 2Bb). β-Catenin was localized on the membrane in fundic tumors (Fig. 2Ad) as well as in normal glands. In contrast, most pyloric tumors harbored β-catenin in cytoplasms or nuclei both in WT and Tg mice (Fig. 2Bd and Cd, respectively) (Table 5). Gastric cancer cells in the pyloric region contained little PAS-positive mucin (Fig. 2Be and Ce), whereas those in fundic area retained AB- and PAS-positive mucin (Fig. 2Ae).

β-Catenin accumulation and gene mutations in gastric tumors. Data for number and frequency of fundic and pyloric tumors demonstrating β-catenin accumulation are summarized in Table 5. β-Catenin accumulation was significantly more common in tumors in pyloric mucosa compared with those in the fundic region in both WT and Tg genotypes ($P < 0.01$).

To analyze β-catenin gene mutation in gastric tumors with β-catenin accumulation, microdissection was performed for corresponding regions from serial paraffin sections. All mutations of β-catenin gene exon 3 were identified in β-catenin accumulating (nuclear/cytoplasmic) regions except for one membranous staining case in group D (Table 6). Mutations of β-catenin gene exon 3 were more frequently observed in the Tg mice of group D than in the Tg mice of group C ($P < 0.05$). Mutation frequency of β-catenin accumulating regions in WT and Tg mice in group C were 18.2% and 21.4%. The corresponding

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Table 3. Histopathological response in gastric musoca of K19-C2mE Tg mice

Groups	Treatments	Genotypes	Effective nos.	Neutrophilis	Lymphocytes	Fundic mucosa thickness (μm)		BrdU labeling index (%)
						Fundic mucosa	Pyloric mucosa	
A	Broth	WT	10	0.33 ± 0.82	0.17 ± 0.41	342.8 ± 38.7	7.46 ± 1.94	12.16 ± 2.22
		Tg	10	0.18 ± 0.40	0.27 ± 0.47	520.8 ± 283.0****	7.56 ± 2.00	12.62 ± 3.01
B	*H. pylori*	WT	10	2.70 ± 0.48*	2.80 ± 0.42*	593.2 ± 111.2*	12.38 ± 1.64*	14.97 ± 3.18
		Tg	10	2.60 ± 0.52*	2.70 ± 0.48*	772.1 ± 214.1*****	13.96 ± 1.76*	14.33 ± 2.69
C	Broth + MNU	WT	12	0 ± 0	0.18 ± 0.40	293.3 ± 88.2	7.32 ± 2.37	12.88 ± 3.65
		Tg	15	0.43 ± 0.76	0.07 ± 0.27	438.2 ± 162.1******	7.70 ± 1.45	12.86 ± 3.11
D	*H. pylori* + MNU	WT	24	1.58 ± 1.02**	2.00 ± 0.83**	441.9 ± 151.8**	13.43 ± 7.18**	15.66 ± 3.78
		Tg	19	2.11 ± 0.68**	2.67 ± 0.69**,***	517.5 ± 129.2	16.95 ± 4.23**,***	16.04 ± 5.46*******

BrdU, 5′-bromo-2′-deoxyuridine; *H. pylori*, *Helicobacter pylori*; MNU, *N*-methyl-*N*-nitrosourea; Tg, Transgenic; WT, wild type.
*$P < 0.01$ *versus* corresponding genotypes in group A; **$P < 0.01$ *versus* corresponding genotypes in group C; ***$P < 0.01$ *versus* WT within group D; ****$P < 0.05$ *versus* WT within group A; *****$P < 0.01$ *versus* WT within group B; ******$P < 0.01$ *versus* WT within group C; *******$P < 0.05$ *versus* Tg in group D. Values for results are expressed as averages ± SD.

Table 4. Relative mRNA expression levels of inflammatory cytokines in gastric mucosa of K19-C2mE mice

Groups	Treatments	Genotypes	Relative mRNA expression levels (range)					
			COX-2	TNF-α	iNOS	IL-1β	IL-6	CXCL14
A	Broth	WT	1.00 (0.55–1.81)	1.00 (0.49–2.02)	1.00 (0.58–1.73)	1.00 (0.31–3.20)	1.00 (0.38–2.63)	1.00 (0.52–1.93)
		Tg	1824.28 (1448.04–2298.28)******	0.68 (0.37–1.24)	0.68 (0.18–2.52)	0.97 (0.37–2.49)	1.22 (0.64–2.29)	0.76 (0.41–1.43)
B	*H. pylori*	WT	2.07 (0.76–5.64)	27.74 (16.21–47.46)*	18.85 (8.56–41.49)*	31.45 (20.52–48.20)*	2.74 (1.83–4.10)**	3.96 (2.94–5.34)*
		Tg	1272.96 (694.86–2332.02)******	42.98 (15.66–117.96)*	38.59 (9.57–155.70)*	32.41 (14.04–74.81)*	2.55 (2.08–3.14)*	5.12 (3.25–8.06)*
C	Broth + MNU	WT	2.00 (1.18–3.39)	5.61 (2.55–12.32)*	4.23 (2.92–6.12)*	8.42 (4.35–16.30)*	1.23 (0.35–4.27)	4.94 (2.21–11.07)*
		Tg	2333.23 (1588.37–3427.38)******	4.81 (2.75–8.43)*	5.98 (3.11–11.51)*	9.61 (4.73–19.55)*	1.59 (0.13–19.12)	6.35 (3.35–12.03)*
D	*H. pylori* + MNU	WT	2.18 (1.21–3.91)	26.63 (8.78–80.76)****	19.08 (5.13–71.02)****	25.18 (10.67–59.45)****	3.22 (1.61–6.44)	4.53 (2.42–8.47)***
		Tg	901.64 (492.31–1651.30)******	61.19 (20.55–182.24)****,******	42.31 (12.94–138.40)****,******	54.03 (19.62–148.80)****,******	3.74 (1.29–10.85)	8.04 (5.09–12.71)***,******

COX-2, cyclooxygenase-2; *H. pylori*, *Helicobacter pylori*; IL-1β, interleukin-1β; IL-6, interleukin-6; iNOS, inducible nitric oxide synthase; MNU, *N*-methl-*N*-nitrosourea; Tg, Transgenic; TNF-α, tumor necrosis factor-α; WT, wild type.
*$P < 0.01$ or **$P < 0.05$ *versus* corresponding genotypes in group A; ***$P < 0.01$ *versus* corresponding genotypes in group B; ****$P < 0.01$ or *****$P < 0.05$ *versus* corresponding genotypes in group C; ******$P < 0.01$ *versus* WT in same groups. Values are expressed as mean. Ranges in parenthesis are expressed as mean – SD to mean + SD.

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Table 5. Number and frequency of β-catenin accumulation in fundic and pyloric tumors

Groups	Treatments	Genotypes	Number (frequency) of β-catenin accumulation	
			Fundic mucosa	Pyloric mucosa
C	Broth + MNU	WT	NA	12/12 (100%)
		Tg	0/4 (0%)	15/15 (100%)*
D	*H. pylori* + MNU	WT	NA	22/24 (91.6%)
		Tg	1/4 (25%)	18/18 (100%)**

H. pylori, Helicobacter pylori; MNU, *N*-methyl-*N*-nitrosourea; NA, not applicable due to no tumors; Tg, Transgenic; WT, wild type.
$^*P < 0.01$ *versus* that of fundic mucosa in Tg within group C; $^{**}P < 0.01$ *versus* that of fundic mucosa in Tg within group D.

figures in group D were 31.6% and 62.5%, respectively. All mutations observed in pyloric tumors were transitions: C→T (9/22 = 41%), G→A (9/22 = 41%), T→C (4/22 = 18%) (Table 7). No mutations were detected in surrounding normal mucosa.

Discussion

In the present study, K19-C2mE Tg mice developed gastric tumors not only in the pyloric mucosa but also the fundic region, whereas WT mice developed tumors only in pyloric areas with the carcinogen treatment but irrelevant to *H. pylori* infection. Furthermore, Tg mice possessed larger tumors even without *H. pylori* infection. On the other hand, Tg mice showed increased tumor multiplicity compared with the corresponding WT mice only with *H. pylori* infection. These findings indicated that transgenes in combination with *H. pylori* infection and subsequent inflammatory response should play important roles in promotion of gastric carcinogenesis in various ways in this mouse model.

Immunohistochemical analysis here demonstrated that tumor cells in the fundic region more markedly express COX-2 than those in the pyloric region. COX-2 expression was predominantly observed in foveolar epithelial cells in K19-C2mE Tg mice. Thus fundic tumors might be derived from foveolar epithelial cells and be more significantly affected by COX-2 expression compared with pyloric tumors. In human gastric neoplasia, proximal gastric tumors are suggested to be specific subtypes of gastric carcinoma based on histological and genetic research.[21,22] K19-C2mE Tg mice may serve as a new animal model for proximal gastric carcinogenesis.

In the present study, *H. pylori* infection did not promote gastric carcinogenesis in the fundic region, and influence could not be evaluated in the pyloric region in terms of cancer incidence. However, there is abundant evidence from rodent gastric cancer models that *H. pylori* infection promotes gastric cancers induced by stomach carcinogens, MNU and *N*-methyl-*N'*-nitroso-*N*-nitrosoguanidine (MNNG),[23–27] although not without exceptions.[28] *H. pylori* infection induced gastritis and caused hyperplasia of the gastric mucosa in the current mouse system, but heterotopic proliferating glands arising with long-term infection of *H. pylori* and considered as high-grade inflammation in the Mongolian gerbil,[29,30] were not observed here. Such lesions are reversible and are considered as regenerative lesions due to excessive cell proliferation. The observations indicate that the influence of *H. pylori* infection may depend on the animal species with clear differences between the mouse and Mongolian gerbil. In addition, host immune responses or *H. pylori* virulence factors may affect gastritis and gastric carcinogenesis.[31,32] By the fact of increased multiplicity of pyloric tumors in *H. pylori*-infected Tg mice, overexpression of COX-2 and mPGES-1 may serve as a better mouse model for mimicking human cases.

Table 6. Localization of β-catenin in gastric tumors and mutation frequency

Groups	Treatments	Genotypes	Animal nos.	Frequency of mice with mutations	β-Catenin mutation frequency in fundic tumors		β-Catenin mutation frequency in pyloric tumors		Surrounding normal mucosa
					Nuclear + Cytoplasmic	Membranous	Nuclear + Cytoplasmic	Membranous	
C	Broth + MNU	WT	10	2/10 (20%)	NA	NA	2/11 (18.2%)	0/7 (0%)	0/7 (0%)
		Tg	10	2/10 (20%)	NA	0/4 (0%)	3/14 (21.4%)	0/10 (0%)	0/6 (0%)
D	*H. pylori* + MNU	WT	10	4/10 (40%)	NA	NA	6/19 (31.6%)	0/10 (0%)	0/7 (0%)
		Tg	10	8/10 (80%)*	0/1 (0%)	0/4 (0%)	10/16 (62.5%)**,***	1/14 (7.1%)	0/7 (0%)

H. pylori, Helicobacter pylori; MNU, *N*-methyl-*N*-nitrosourea; NA, not applicable due to no tumors; Tg, Transgenic; WT, wild type.
$^*P < 0.05$ *versus* Tg in group C; $^{**}P < 0.05$ *versus* Tg in group C; $^{***}P < 0.05$ *versus* Tg in 'membranous' in within group D.

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Table 7. Mutation of β-catenin exon 3 in gastric tumors

Groups	Treatments	Genotypes	Mice nos.	Sample nos.	Tumor location	β-Catenin localization	Mutations	Amino acid changes	Events
C	Broth + MNU	WT	W-6	T4	Pylorus	Nuclear/Cytoplasmic	codon 41: ACC→ATC	Thr→Ile	Transition
			W-11	T9	Pylorus	Nuclear/Cytoplasmic	codon 34: GGA→GAA	Gly→Glu	Transition
		Tg	T-1	T12	Pylorus	Nuclear/Cytoplasmic	codon 32: GAT→AAT	Asp→Asn	Transition
				T13	Pylorus	Nuclear/Cytoplasmic	codon 32: GAT→AAT	Asp→Asn	Transition
			T-12	T22	Pylorus	Nuclear/Cytoplasmic	codon 34: GGA→GAA	Gly→Glu	Transition
D	*H. pylori* + MNU	WT	W-8	T27	Pylorus	Nuclear/Cytoplasmic	codon 32: GAT→AAT	Asp→Asn	Transition
			W-11	T75	Pylorus	Nuclear/Cytoplasmic	codon 35: ATC→ATT	Silent	Transition
				T75	Pylorus	Nuclear/Cytoplasmic	codon 45: TCC→TTC	Ser→Phe	Transition
			W-23	T85	Pylorus	Nuclear/Cytoplasmic	codon 41: ACC→ATC	Thr→Ile	Transition
			W-24	T34	Pylorus	Nuclear/Cytoplasmic	codon 33: TCT→CCT	Ser→Pro	Transition
				T35	Pylorus	Nuclear/Cytoplasmic	codon 33: TCT→CCT	Ser→Pro	Transition
		Tg	T-2	T37	Pylorus	Nuclear/Cytoplasmic	codon 41: ACC→ATC	Thr→Ile	Transition
				T38	Pylorus	Nuclear/Cytoplasmic	codon 41: ACC→ATC	Thr→Ile	Transition
				T39	Pylorus	Membranous	codon 41: ACC→ATC	Thr→Ile	Transition
			T-4	T40	Pylorus	Nuclear/Cytoplasmic	codon 32: GAT→AAT	Asp→Asn	Transition
				T41	Pylorus	Nuclear/Cytoplasmic	codon 41: ACC→ATC	Thr→Ile	Transition
			T-5	T88	Pylorus	Nuclear/Cytoplasmic	codon 32: GAT→AAT	Asp→Asn	Transition
			T-6	T90	Pylorus	Nuclear/Cytoplasmic	codon 33: TCT→CCT	Ser→Pro	Transition
			T-16	T158	Pylorus	Nuclear/Cytoplasmic	codon 32: GAT→AAT	Asp→Asn	Transition
			T-17	T147	Pylorus	Nuclear/Cytoplasmic	codon 32: GAT→AAT	Asp→Asn	Transition
			T-18	T92	Pylorus	Nuclear/Cytoplasmic	codon 41: ACC→ATC	Thr→Ile	Transition
			T-19	T149	Pylorus	Nuclear/Cytoplasmic	codon 41: ACC→ATC	Thr→Ile	Transition

H. pylori, Helicobacter pylori; MNU, *N*-methyl-*N*-nitrosourea; Tg, Transgenic; WT, wild type.

For further analysis of factors promoting gastric carcinogenesis, we investigated the expression of inflammatory cytokines in gastric mucosa. In the present study, expression of those such as TNF-α, iNOS, IL-1β, and CXCL14 were significantly increased in Tg mice with *H. pylori* infection and MNU treatment. Among them, CXCL14 has been known to selectively attract monocytes, where PGE_2 up-regulates their responsiveness.[33] The combination of these factors may contribute to the participation of macrophages in increased tumorigenesis in Tg mice with *H. pylori* infection plus MNU treatment. We previously demonstrated that the severity of chronic gastritis, characterized by high-level expression of IL-1β, TNF-α, COX-2, and iNOS was concerned with glandular gastric carcinogenesis in *H. pylori*-infected Mongolian gerbils.[34] Thus, in Tg mice with *H. pylori* infection a higher level of inflammatory cytokines may be induced that eventually promotes gastric carcinogenesis.

The Tg mice feature increased PGE_2 synthesis due to overexpressed COX-2/mPGES-1 in gastric mucosa.[4] PGE_2 exerts its biological effects by binding to four isoform receptors, EP_1, EP_2, EP_3, and EP_4,[35,36] categorized in the family of seven transmembrane G protein coupled rhodopsin-type receptors. Accumulating evidence indicates that PGE_2 promotes tumor growth by stimulating EP receptor signaling with subsequent enhancement of cell proliferation, promotion of angiogenesis, and inhibition of apoptosis.[37] Previous reports based on mouse studies demonstrated that EP_1, EP_2, and EP_4 receptors play important roles in colon carcinogenesis.[38–41] Furthermore, expression of EP_1, EP_2, and EP_4 receptors has been found to be elevated in mouse mammary tumors as well as colon cancers.[42] EP_3 receptor activation was furthermore suggested to contribute to breast cancer progression.[43] These observations indicate that the expression pattern of EP receptors in cancer cells might determine the potential of PGE_2 to drive tumor progression. Although the expression level of EP receptors in gastric cancers was unclear in the present study, EP receptor signaling stimulated by PGE_2 might have influenced gastric carcinogenesis. It should be stressed that PGE_2 transactivates epidermal growth factor receptor (EGFR) and triggers PI3K/Akt signaling,[44–46] and Ras/MEK/ERKs,[47] pathways in gastric epithelial and colon cancer cells *in vitro* as well as rat gastric mucosa *in vivo*.[46]

To analyze the differences between present mouse gastric tumors developed in fundic and pyloric mucosas, we investigated β-catenin activation, which was suggested to play an important role in gastric carcinogenesis. Immunohistochemical analysis here showed that β-catenin activation characterized by its intracellular accumulation was frequently observed in tumors in the pyloric region, in contrast to those in the fundic region, indicating the unnecessariness of the Wnt pathway. Conversely, pyloric tumorigenesis might be promoted by Wnt activation. In a rat model, a type of adenocarcinoma resembling foveolar epithelium showed nuclear accumulation of cyclin D1 without β-catenin activation,[48] whereas β-catenin was accumulated in cytoplasms or nuclei in the majority of less-differentiated adenocarcinomas.[20] Since fundic tumors were histologically classified as being of the foveolar type and pyloric ones were classified as the less differentiated in this experiment, oncogene activation could depend on cell/tissue differentiation or vice versa.[49] Similarly, mucin expression characterized by AB-PAS staining was observed in fundic tumors as in the hyperplastic fundic tumors in the previous report;[4] pyloric tumors, however, lost most of those mucin production.

Several previous studies of gastric carcinogenesis models in rodents such as the mouse, rat, and Mongolian gerbil have indicated that β-catenin activation plays an important role in gastric carcinogenesis.[20,50–52] In the present study, this was more frequently observed than in previous reports (rat, 18.2%; mouse, 12.5%; and Mongolian gerbil, 2.2%).[20,50,51] Such variation in the frequency of β-catenin activation might be caused by differences in experimental design such as the experimental period, chemical carcinogen applied, or type of experimental animal.

In rat and Mongolian gerbil models, mutations of β-catenin gene in exon 3 have been identified in codons 34, 41, and 45 at GSK-3β phosphorylation sites, and are significantly associated with nuclear β-catenin accumulation.[20,51] In human gastric cancers,

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gene alternations have been found in the same sites including codons 29, 37, 41, and 47 as well as in adjacent sites at codons 28, 32, 34, 36, 38, 39, and 48.[12,13,19,53] In the present experiment, the mutation spectrum was codons 32, 33, 34, 35, 41, and 45, consistent with the previous reports. Furthermore, β-catenin mutations were particularly frequent in Tg mice with *H. pylori* infection. Tumor multiplicity was also increased in Tg mice with *H. pylori* infection, indicating the possibility that *H. pylori* infection in addition to COX-2/mPGES-1 expression might contribute to progression of gastric adenocarcinomas though β-catenin gene alternations at least in part. In humans, stomach cancers with intestinal differentiation markers feature more β-catenin mutations compared to those with gastric markers.[19] Furthermore, intestinal markers may be induced only in stomach tumors in *H. pylori*-infected gerbils.[54] Thus, alteration of β-catenin could be related to *H. pylori* infection, although further work is needed to reveal interactions between these two factors.

β-Catenin accumulation without β-catenin gene mutations was detected in some tumors in the present study, indicating involvement of other alterations of Wnt pathway regulatory genes. Indeed, there have been a large number of previous reports suggesting that *APC* gene mutation,[17,55–61] APC loss of heterozygosity,[59] over-expression of various Wnt ligands, and altered frizzled receptors[62–64] may be involved in β-catenin activation. Furthermore, degradation of E-cadherin and microsatellite instability might also be responsible for β-catenin accumulation. In one rodent model, the $APC^{Min/+}$ mouse which harbors a nonsense mutation at codon 850 of the *APC* gene, gastric tumors frequently develop with *APC* loss of heterozygosity.[50]

In conclusion, the present study indicated that over-expression of COX-2/mPGES-1 promotes gastric carcinogenesis, especially in the fundic region, further showing the K19-C2mE Tg mouse to be a new animal model for proximal gastric carcinogenesis. Furthermore Tg mice developed multiple tumors in the pyloric region with *H. pylori* infection partly with β-catenin gene mutation and activation. This indicates the risk of multiple or metachronous gastric cancers also in human cases with *H. pylori* infection and supports the idea to eradicate the bacterium or to suppress inflammatory response for the prevention of secondary malignancies.[65]

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References

1 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.

2 Kuipers EJ, Uyterlinde AM, Pena AS *et al.* Long-term sequelae of *Helicobacter pylori* gastritis. *Lancet* 1995; **345**: 1525–8.

3 Sung JJ, Leung WK, Go MY *et al.* Cyclooxygenase-2 expression in *Helicobacter pylori*-associated premalignant and malignant gastric lesions. *Am J Pathol* 2000; **157**: 729–35.

4 Oshima H, Oshima M, Inaba K, Taketo MM. Hyperplastic gastric tumors induced by activated macrophages in COX-2/mPGES-1 transgenic mice. *Embo J* 2004; **23**: 1669–78.

5 Hisatsune J, Yamasaki E, Nakayama M *et al. Helicobacter pylori* VacA enhances prostaglandin E2 production through induction of cyclooxygenase 2 expression via a p38 mitogen-activated protein kinase/activating transcription factor 2 cascade in AZ-521 cells. *Infect Immun* 2007; **75**: 4472–81.

6 Tanigawa T, Watanabe T, Hamaguchi M *et al.* Anti-inflammatory effect of two isoforms of COX in *H. pylori*-induced gastritis in mice: possible involvement of PGE2. *Am J Physiol Gastrointest Liver Physiol* 2004; **286**: G148–56.

7 Wang MT, Honn KV, Nie D. Cyclooxygenases, prostanoids, and tumor progression. *Cancer Metastasis Rev* 2007; **26**: 525–34.

8 Oshima H, Matsunaga A, Fujimura T, Tsukamoto T, Taketo MM, Oshima M. Carcinogenesis in mouse stomach by simultaneous activation of the Wnt signaling and prostaglandin E2 pathway. *Gastroenterology* 2006; **131**: 1086–95.

9 Kikuchi A. Modulation of Wnt signaling by Axin and Axil. *Cytokine Growth Factor Rev* 1999; **10**: 255–65.

10 Akiyama T. Wnt/beta-catenin signaling. *Cytokine Growth Factor Rev* 2000; **11**: 273–82.

11 Sasaki Y, Morimoto I, Kusano M *et al.* Mutational analysis of the beta-catenin gene in gastric carcinomas. *Tumour Biol* 2001; **22**: 123–30.

12 Woo DK, Kim HS, Lee HS, Kang YH, Yang HK, Kim WH. Altered expression and mutation of beta-catenin gene in gastric carcinomas and cell lines. *Int J Cancer* 2001; **95**: 108–13.

13 Clements WM, Wang J, Sarnaik A *et al.* Beta-Catenin mutation is a frequent cause of Wnt pathway activation in gastric cancer. *Cancer Res* 2002; **62**: 3503–6.

14 Ohene-Abuakwa Y, Noda M, Perenyi M *et al.* Expression of the E-cadherin/catenin (alpha-, beta-, and gamma-) complex correlates with the macroscopic appearance of early gastric cancer. *J Pathol* 2000; **192**: 433–9.

15 Miyazawa K, Iwaya K, Kuroda M *et al.* Nuclear accumulation of beta-catenin in intestinal-type gastric carcinoma: correlation with early tumor invasion. *Virchows Arch* 2000; **437**: 508–13.

16 Tong JH, To KF, Ng EK *et al.* Somatic beta-catenin mutation in gastric carcinoma – an infrequent event that is not specific for microsatellite instability. *Cancer Lett* 2001; **163**: 125–30.

17 Ebert MP, Fei G, Kahmann S *et al.* Increased beta-catenin mRNA levels and mutational alterations of the APC and beta-catenin gene are present in intestinal-type gastric cancer. *Carcinogenesis* 2002; **23**: 87–91.

18 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.

19 Ogasawara N, Tsukamoto T, Mizoshita T *et al.* Mutations and nuclear accumulation of beta-catenin correlate with intestinal phenotypic expression in human gastric cancer. *Histopathology* 2006; **49**: 612–21.

20 Tsukamoto T, Yamamoto M, Ogasawara N *et al.* Beta-Catenin mutations and nuclear accumulation during progression of rat stomach adenocarcinomas. *Cancer Sci* 2003; **94**: 1046–51.

21 Abraham SC, Nobukawa B, Giardiello FM, Hamilton SR, Wu TT. Fundic gland polyps in familial adenomatous polyposis: neoplasms with frequent somatic adenomatous polyposis coli gene alterations. *Am J Pathol* 2000; **157**: 747–54.

22 Tajima Y, Yamazaki K, Makino R *et al.* Differences in the histological findings, phenotypic marker expressions and genetic alterations between adenocarcinoma of the gastric cardia and distal stomach. *Br J Cancer* 2007; **96**: 631–8.

23 Shimizu N, Inada K, Nakanishi H *et al. Helicobacter pylori* infection enhances glandular stomach carcinogenesis in Mongolian gerbils treated with chemical carcinogens. *Carcinogenesis* 1999; **20**: 669–76.

24 Sugiyama A, Maruta F, Ikeno T *et al. Helicobacter pylori* infection enhances N-methyl-N-nitrosourea-induced stomach carcinogenesis in the Mongolian gerbil. *Cancer Res* 1998; **58**: 2067–9.

25 Shimizu N, Inada KI, Tsukamoto T *et al.* New animal model of glandular stomach carcinogenesis in Mongolian gerbils infected with *Helicobacter pylori* and treated with a chemical carcinogen. *J Gastroenterol* 1999; **34** (Suppl 11): 61–6.

26 Shimizu N, Kaminishi M, Tatematsu M *et al. Helicobacter pylori* promotes development of pepsinogen-altered pyloric glands, a preneoplastic lesion of glandular stomach of BALB/c mice pretreated with N-methyl-N-nitrosourea. *Cancer Lett* 1998; **123**: 63–9.

27 Nam KT, Hahm KB, Oh SY *et al.* The selective cyclooxygenase-2 inhibitor nimesulide prevents *Helicobacter pylori*-associated gastric cancer development in a mouse model. *Clin Cancer Res* 2004; **10**: 8105–13.

28 Nakamura Y, Sakagami T, Yamamoto N *et al. Helicobacter pylori* does not promote N-methyl-N-nitrosourea-induced gastric carcinogenesis in SPF C57BL/6 mice. *Jpn J Cancer Res* 2002; **93**: 111–16.

29 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.

30 Tatematsu M, Tsukamoto T, Mizoshita T. Role of *Helicobacter pylori* in gastric carcinogenesis: the origin of gastric cancers and heterotopic proliferative glands in Mongolian gerbils. *Helicobacter* 2005; **10**: 97–106.

31 Hatakeyama M. SagA of CagA in *Helicobacter pylori* pathogenesis. *Curr Opin Microbiol* 2008; **11**: 30–7.

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