Combination of Sulindac and Antimicrobial Eradication of Helicobacter pylori Prevents Progression of Gastric Cancer in Hypergastrinemic INS-GAS Mice

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Abstract

Helicobacter pylori infection causes severe dysplasia manifested as gastrointestinal intraepithelial neoplasia (GIN) after 28 weeks post–H. pylori infection (WPI) in cancer-prone, hypergastrinemic male INS-GAS mice. We examined the efficacy of the nonsteroidal anti-inflammatory drug sulindac (400 ppm in drinking water) alone, the CCK2/gastrin receptor antagonist YM022 (45 mg/kg/wk) alone, and sulindac or YM022 combined with H. pylori eradication therapy to prevent H. pylori–associated gastric cancer in male INS-GAS mice. Treatments started at 22 WPI, and mice were euthanized at 28 WPI. In uninfected mice, all treatments significantly delayed development of spontaneous GIN (P < 0.05). In H. pylori–infected mice, sulindac alone or YM022 alone had no protective effect on H. pylori–associated GIN. Importantly, sulindac exacerbated the severity of H. pylori–associated gastritis despite decreased gastric prostaglandin E2 levels. However, sulindac combined with H. pylori antimicrobial eradication reduced the incidence of GIN (P < 0.05), whereas YM022 combined with antimicrobial eradication did not reduce GIN. In infected mice, sulindac or YM022 treatment did not alter gastric expression of the proinflammatory cytokines Ifn-γ and Tnf-α and mucosal cell proliferation. Sulindac or YM022 combined with antimicrobial eradication down-regulated mRNA levels of Ifn-γ and Tnf-α and mucosal cell proliferation (P < 0.05). We conclude that sulindac enhances H. pylori gastritis and may promote inflammation-mediated gastric carcinogenesis. The combination of sulindac and antimicrobial H. pylori eradication was beneficial for reducing proinflammatory cytokine mRNA in the stomach and preventing progression from severe dysplasia to gastric cancer in H. pylori–infected INS-GAS mice. [Cancer Res 2009;69(20):8166–74]

Introduction

Based on experimental animal models and epidemiologic evidence, Helicobacter pylori infection causes persistent chronic gastritis, which in susceptible individuals may progress to atrophy, intestinal metaplasia, dysplasia, and finally intestinal-type gastric cancer (1–4). H. pylori infection resulted in overexpression of cyclooxygenase-2 (Cox-2) in primary gastric cancer and gastric cancer cell lines of human and mouse gastric epithelial cells (5, 6). Double-transgenic mice that constitutively expressed Cox-2 and prostaglandin E synthase-1 (Pges-1) in gastric epithelial cells had higher levels of prostaglandin E2 (PGE2) in the stomach and spontaneously develop macrophage infiltration, metaplasia, and gastric adenocarcinoma. This Cox-2/PGE2–related gastric cancer was suppressed by a Cox-2 inhibitor, NS-398 (7), suggesting that Cox-2/PGE2 pathway may contribute to Helicobacter–associated gastric carcinogenesis. Recent studies suggested a positive association between hypergastrinemia, H. pylori infection, and gastric cancer in humans and mice (8–11). Hypergastrinemia and Helicobacter infection synergistically promoted gastric cancer in male transgenic INS-GAS mice overexpressing amidated gastrin (8, 9, 11). Because decades of H. pylori infection are a prerequisite for gastric cancer development in susceptible hosts (1), chemoprevention is one of the promising approaches in gastric cancer prevention.

Because overexpression of Cox-2 and production of PGE2 are strongly associated with increased proliferation and reduced apoptosis in gastrointestinal epithelial tumor cells (12), nonsteroidal anti-inflammatory drugs (NSAID) that inhibit Cox activities and production of PGE2 are among the most widely tested compounds for cancer chemoprevention (13, 14). Inhibition of Cox activities decreased cell proliferation in gastric and intestinal cell lines that constitutively expresses Cox-2 (15, 16). Animal models showed that the nonselective Cox inhibitor sulindac prevents oral-esophageal cancer and colon cancer (17, 18). Epidemiologic studies also associate consumption of aspirin or other NSAIDs with a reduced risk of colorectal cancer (19, 20). In addition, clinical trials confirmed that sulindac and the selective Cox-2 inhibitor celecoxib inhibit the number and growth of adenomatous polyps in patients with familial adenomatous polyposis (21, 22). A lower risk of gastric cancer has been associated with NSAIDs in a dose-dependent manner (23). Considering the association between Cox-2/PGE2 pathway and Helicobacter–associated gastric carcinogenesis, NSAIDs have been proposed as candidates for chemoprevention of gastric cancer. However, recent data indicate that suppression of PGE2 by Cox-2 inhibition enhanced Th1 proinflammatory immune responses in H. pylori–infected humans (24, 25); this raises the possibility that NSAIDs may increase H. pylori–associated gastritis and enhance inflammation-mediated gastric carcinogenesis (2). It also has been shown that long-term treatment of CCK2/gastrin receptor antagonist YF476 prevented the development of Helicobacter–associated gastric cancer in INS-GAS mice (26, 27), suggesting that the gastrin signaling pathway provides another...
potential target for cancer chemoprevention. However, the effect of short-term CCK2/gastrin receptor antagonist treatment has not been analyzed. Long-term treatment of sulindac did not cause tissue injury of gastric mucosa of mice (18). A recent study showed that gastric PGE2 in H. pylori infection was mainly derived from Cox-1, and the selective Cox-2 inhibitor rofecoxib did not suppress PGE2 production or gastric epithelial proliferation, biomarkers of carcinogenesis (28).

We examined the chemopreventive effects of the nonselective Cox inhibitor sulindac and CCK2/gastrin receptor antagonist YM022 (29), an analogue of YF476 (26), alone or in combination with H. pylori antimicrobial eradication during the chronic stage of H. pylori infection in male INS-GAS mice.

Materials and Methods

Mice. Specific pathogen-free (including Helicobacter spp.) male INS-GAS mice on a FVB/N background were maintained in an Association for Assessment and Accreditation of Laboratory Animal Care–accredited facility housed in microisolator cages and given a commercial rodent diet (Prolab 3000) and water ad libitum. The animal protocol was approved by the MIT Committee on Animal Care.

Experimental design. Fifty-four 6- to 8-week-old male mice were infected by oral gavage with 0.2 ml H. pylori (SS1 strain) on alternate days for a total of three doses (30). The H. pylori inoculum was adjusted with PBS to A600 = 1.0 (30). Helicobacter-uninfected mice were sham-dosed with 0.2 ml PBS. Antimicrobial therapy used to eradicate H. pylori consisted of omeprazole (400 μmol/kg/d; Sigma-Aldrich), metronidazole (14.2 mg/kg/d; Sigma-Aldrich), and clarithromycin (7.15 mg/kg/d; Abbott) twice a day for 7 days. This regimen has been used successfully in eradicating H. pylori from experimentally infected mice (31, 32). Antimicrobial H. pylori therapy was administered at 22 weeks post–H. pylori infection (WPI). Sulindac was dissolved in buffer (4 mmol/L NaHPO4, pH 7.4) at the final concentration of 400 ppm, given ad libitum, and changed daily from 22 WPI (18). YM022 was dissolved in polyethylene glycol 300 (Sigma) by stirring for 24 h at room temperature, and 45 mg/kg was injected i.c. once a week starting at 22 WPI (27). Mice were euthanized at 28 WPI.

Tissue collection and gastric pH measurement. Mice were fasted overnight (8-14 h) before necropsy. Following CO2 asphyxiation, blood was drawn from the tail vein and stored at −70°C. Total RNA was extracted with Trizol reagent (Invitrogen). cDNA was synthesized from 5 μg total RNA with High-Capacity cDNA Archive kit. mRNA levels of Hif-1, Trf-a, Il-4, Cox-1 (or Ptgs1; Applied Biosystems), Cox-2 (or Ptgs2), and glyceraldehyde-3-phosphate dehydrogenase (Gapdh) were quantified with TaqMan gene expression assays using an ABI Prism Sequence Detection System 7700 (Applied Biosystems). mRNA levels of each gene were normalized to the mRNA level of internal control Gapdh and compared with the data of uninfectected mice using the ∆∆CT method (User Bulletin 2; Applied Biosystems).

Statistical analysis. Gastric lesion scores and Ki-67 LI for proliferation indices were compared using the Mann-Whitney U test. PGE2 levels, expression levels of cytokines, and IgG titers were compared using the Student’s t test. Incidences of low-grade and high-grade GIN in the treatment groups were compared with controls by χ2 test and Fisher’s exact test using commercial software (GraphPad Prism 4.0; GraphPad Software). Data were presented as mean ± SE.

Results

Effect of chemopreventive therapies on gastric pH in uninfected and H. pylori–infected INS-GAS mice. To assess the effect of H. pylori infection with or without treatment on the gastric acidity of INS-GAS mice, the pH of gastric aspirate was measured. Untreated uninfected INS-GAS mice had a gastric pH 4.76 ± 2. Among H. pylori–uninfected mice, gastric pH was reduced by sulindac (3.37 ± 0.16; P < 0.001), and sulindac and antimicrobial therapy also reduced gastric pH (3.85 ± 0.38; P = 0.07; Supplementary Fig. S1). Uninfected mice that received sulindac or sulindac and antimicrobial therapy had comparable gastric pH (P = 0.21). In contrast, uninfected mice that received gastrin receptor antagonist YM022 or YM022 and antimicrobial therapy did not alter gastric pH (4.72 ± 0.25 and 4.59 ± 0.2, respectively).

H. pylori infection and antimicrobial eradication were confirmed by quantitative PCR at necropsy. H. pylori was successfully eradicated in all mice (100%) that received antimicrobial therapy at 22 WPI (data not shown). H. pylori infection significantly increased the gastric pH in INS-GAS mice (6.28 ± 0.12; P < 0.001). Among H. pylori–infected mice, the gastric pH was reduced by sulindac (4.96 ± 0.22) and sulindac and antimicrobial therapy (4.59 ± 0.22; both P < 0.001). H. pylori–infected mice that received sulindac or sulindac and antimicrobial therapy had a similar gastric pH (P = 0.24). Infected mice that received YM022 had a gastric pH (6.35 ± 0.05) comparable with that in untreated infected mice. However, YM022 and antimicrobial therapy reduced the gastric pH (5.55 ± 0.25) in infected mice when compared with untreated infected mice (P = 0.07) or infected mice that received YM022 (P < 0.01).

Sulindac and YM022 inhibit gastric preneoplasic in uninfected INS-GAS mice. H. pylori–infected mice developed

A. Rustagi, personal communication.
mild inflammation, epithelial defects, moderate hyperplasia, and severe oxyntic atrophy, intestinal metaplasia, and dysplasia at ages 34 to 36 weeks (equivalent to 28 WPI; Figs. 1A and 2). Of the uninfected mice, 66.67% developed low-grade GIN and none developed high-grade GIN. Sulindac treatment started at ages 28 to 30 weeks (equivalent to 22 WPI) significantly reduced premalignant lesions (P < 0.01) and incidence of gastric cancer (P < 0.05) in uninfected mice (Fig. 2). Low-grade GIN was observed in 10% of the uninfected INS-GAS mice that received sulindac, whereas high-grade GIN was not diagnosed. Sulindac and antimicrobial therapy started at ages 28 to 30 weeks also significantly reduced the severity of premalignant (P < 0.05) and
dysplasia (D). Tissues were stained with H&E. Bar, 400 μm.

Figure 1. Gastric histology in mice. At ages 32 to 34 wk, uninfected male mice exhibited spontaneous gastric epithelial dysplasia with inflammation (A). H. pylori infection significantly increased inflammation, oxyntic atrophy, foveolar hyperplasia, and dysplasia in the corpus of age-matched male mice at 28 WPI (B). Sulindac treatment exacerbated inflammation but not hyperplasia or dysplasia in H. pylori–infected mice (C). H. pylori antimicrobial therapy and sulindac significantly reduced inflammation, oxyntic atrophy, foveolar hyperplasia, and dysplasia (D). Tissues were stained with H&E. Bar, 400 μm.

Figure 2. Gastric histologic scores in sulindac groups: inflammation (A), atrophy (B), dysplasia (C), and GIN (D). Sulindac and a combination of sulindac and antimicrobial therapy reduced incidence of both low-grade and high-grade GIN in H. pylori–uninfected mice (P < 0.05 and P < 0.001, respectively), whereas the combination of sulindac and H. pylori eradication reduced gastric cancer incidence in H. pylori–infected mice (P < 0.05). *, P < 0.05; **, P < 0.01; ***, P < 0.001.
malignant ($P < 0.05$) lesions in the uninfected INS-GAS mice. None of the uninfected mice that received the combination of sulindac and antimicrobial therapy developed GIN.

YM022 treatment slightly reduced dysplasia ($P = 0.079$) and the incidence of gastric cancer ($P = 0.087$) in uninfected mice (Fig. 3). Low-grade GIN was diagnosed in 25% of the YM022-treated uninfected mice. YM022 and antimicrobial therapy resulted in less severe dysplasia ($P < 0.01$) and incidence gastric cancer ($P < 0.01$) in uninfected mice. Additionally, uninfected mice that received YM022 or the combination of YM022 and antimicrobial therapy had comparable severity of gastric lesions ($P > 0.05$).

**H. pylori infection promotes gastric carcinogenesis.** *Helicobacter* infection promotes gastric carcinogenesis in mice (9, 11). *H. pylori*-infected mice developed more severe gastric inflammation ($P = 0.065$) and epithelial defects ($P < 0.01$) than uninfected mice (Fig. 2). *H. pylori* infection also promoted the development of gastric cancer in all infected mice.

**Sulindac increases gastric preneoplasia, whereas sulindac and antimicrobial therapy reduces gastric carcinogenesis in *H. pylori*-infected mice.** In *H. pylori*-infected mice, sulindac exacerbated the severity of inflammation ($P < 0.01$), oxyntic atrophy ($P < 0.05$), and intestinal metaplasia ($P < 0.05$; Figs. 1C and 2). Epithelial hyperplasia was reduced by sulindac ($P < 0.01$; data not shown). The severity of dysplasia and the incidence of gastric cancer were comparable between untreated infected mice and infected mice that received sulindac. All infected mice that received sulindac developed gastric cancer with both low-grade and high-grade GIN being observed in a *H. pylori*-infected INS-GAS mice (80% and 20%, respectively). Compared with untreated *H. pylori*-infected mice, infected mice that received sulindac and antimicrobial therapy had significantly less severe oxyntic atrophy ($P < 0.05$) and a lower incidence of gastric cancer ($P < 0.05$; Figs. 1D and 2). Gastric cancer was observed in 64% (38% high-grade and 27% low-grade GIN) of infected mice that received sulindac and antimicrobial therapy. The severity of inflammation, epithelial defects, hyperplasia, intestinal metaplasia, and dysplasia were comparable between untreated infected mice and infected mice that received sulindac and antimicrobial therapy. Additionally, when compared with *H. pylori*-infected mice that received sulindac, severity of inflammation ($P < 0.001$), oxyntic atrophy ($P < 0.001$), and intestinal metaplasia ($P < 0.01$) and incidence of gastric cancer ($P < 0.01$) were significantly reduced in infected mice that received the combination therapy.

Compared with untreated *H. pylori*-infected mice, infected mice that received YM022 or a combination of YM022 and antimicrobial therapy showed similar pathology scores (Fig. 3). Gastric cancer developed in all mice that received YM022 and antimicrobial therapy. Low-grade and high-grade GIN were observed in 75% and 25% of infected mice that received YM022 and in 44% and 56% of infected mice that received YM022 and antimicrobial therapy, respectively. YM022 or YM022 and antimicrobial therapy had no significant effect on gastric cancer incidence in *H. pylori*-infected mice.

**Expression of proinflammatory *Ifn-γ* and *Tnf-α*, *Cox-1*, and *Cox-2* in the stomach is up-regulated by sulindac but down-regulated by sulindac and antimicrobial therapy.** Among *H. pylori*-uninfected mice, sulindac significantly up-regulated gastric mRNA levels of *Ifn-γ* and *Tnf-α* ($P < 0.05$; Fig. 4). mRNA levels of *Ifn-γ* were down-regulated in mice that received sulindac and antimicrobial therapy compared with uninfected mice that received sulindac ($P < 0.05$); however, the gastric *Ifn-γ* mRNA levels in mice that received sulindac and antimicrobial therapy were still higher than those in the untreated mice ($P < 0.05$). *H. pylori*-uninfected mice that received combination therapy had similar gastric *Tnf-α* expression compared with untreated mice or mice that received sulindac. In contrast, *Cox-1* and *Cox-2*
expression levels were not altered by sulindac or the combination therapy. Expression levels of proinflammatory cytokines and Cox enzymes were not changed in uninfected mice by YM022 or the combination of YM022 and antimicrobial therapy. Additionally, gastric II-4 mRNA levels were not altered by sulindac or YM022, alone or combined with antimicrobial therapy, respectively.

*H. pylori* infection up-regulated gastric mRNA levels of *Ifn-γ* and *Tnf-z* in male INS-GAS mice (*P* < 0.01; Fig. 5). *H. pylori* infection, however, had no effect on mRNA levels of *Cox-1*, *Cox-2*, and II-4 in the stomach of mice. Compared with untreated *H. pylori*-infected mice, infected mice that received sulindac, infected mice that received sulindac and antimicrobial therapy had down-regulated mRNA levels when compared with untreated infected mice. Compared with untreated and antimicrobial therapy. Additionally, sulindac and antimicrobial therapy had lower gastric PGE2 levels (1.62 ± 0.34 ng/mg protein; *P* = 0.09) than untreated mice. Compared with untreated infected mice, gastric PGE2 levels were slightly higher in mice that received YM022 (4.33 ± 0.71 ng/mg protein; *P* = 0.17) or YM022 and antimicrobial therapy (5.51 ± 0.64 ng/mg protein; *P* < 0.05).

*Sulindac reduces gastric PGE2 levels in INS-GAS mice.* Among *H. pylori*-uninfected mice, sulindac reduced gastric PGE2 levels (1.09 ± 0.27 ng/mg protein) compared with untreated mice (3.03 ± 0.59 ng/mg protein; *P* < 0.01; Fig. 4D). Mice that received sulindac and antimicrobial therapy had lower gastric PGE2 levels (1.62 ± 0.34 ng/mg protein; *P* = 0.09) than untreated mice. Compared with untreated infected mice, gastric PGE2 levels were slightly higher in mice that received YM022 (4.33 ± 0.71 ng/mg protein; *P* = 0.17) or YM022 and antimicrobial therapy (5.51 ± 0.64 ng/mg protein; *P* < 0.05). *H. pylori*-infected INS-GAS mice had significantly higher gastric PGE2 levels (9.73 ± 0.81 ng/mg protein; *P* < 0.01) when compared with uninfected mice. *H. pylori*-induced gastric PGE2 levels were inhibited by sulindac (1.29 ± 0.48 ng/mg protein; *P* < 0.01; Fig. 5D).

Infected mice receiving sulindac and antimicrobial therapy had significantly lower gastric PGE2 levels (3.47 ± 0.76 ng/mg protein; *P* < 0.01) than untreated infected mice. YM022 alone reduced gastric PGE2 levels (6.83 ± 0.81 ng/mg protein; *P* < 0.05) in *H. pylori*-infected mice. YM022 and antimicrobial therapy did...
not alter gastric PGE₂ levels (9.49 ± 0.93 ng/mg protein) when compared with untreated infected mice.

**Sulindac and antimicrobial therapy decreased Th1-associated, *H. pylori*-specific IgG2a antibody responses.** *H. pylori* infection significantly induced *H. pylori*-specific, Th1-associated IgG2a responses in mice (P < 0.001; Supplementary Fig. S2A). Sulindac treatment did not alter *H. pylori*-infected IgG2a levels in *H. pylori*-infected mice (P = 0.09). Infected mice that received sulindac and antimicrobials had *H. pylori*-specific IgG2a levels comparable with untreated infected mice. Additionally, *H. pylori*-specific IgG2a levels in infected mice that received the sulindac and antimicrobial therapy were significantly lower than those in infected mice that received sulindac (P < 0.05). In contrast, YM022 alone or YM022 and antimicrobial therapy did not alter *H. pylori*-specific IgG2a levels in infected mice. *H. pylori* infection also induced *H. pylori*-specific, Th2-associated IgG1 responses in mice (P < 0.001; Supplementary Fig. S2B). Treatment modalities in *H. pylori*-infected mice had no significant effect on *H. pylori*-specific IgG1 levels compared with untreated infected mice.

**Gastric mucosal cell proliferation was reduced by a combination of antimicrobial therapy and sulindac or YM022.** Proliferating epithelial cells detected by Ki-67 immunohistochemical staining were observed in the isthmus regions of corpus mucosa in uninfected mice (Fig. 6B). In untreated *H. pylori*-infected mice, proliferating cells in the corpus expanded from the isthmus regions to hypertrrophic foveolar regions, and the Ki-67 LIIs were higher than in uninfected mice (P < 0.01; Fig. 6A). Among *H. pylori*-infected mice, proliferating cells were observed in the hypertrrophic foveolar regions of the corpus in those mice receiving sulindac or YM022. The distribution of proliferative cells was mainly in the isthmus region of the corpus mucosa in infected mice receiving combination of sulindac or YM022 and *H. pylori* eradication. Ki-67 LI positively correlated with severity of gastric pathology and GIN; Ki-67 LI in *H. pylori*-infected mice receiving sulindac or YM022 was similar to untreated *H. pylori*-infected mice. Infected mice receiving the combination of sulindac or YM022 and *H. pylori* antimicrobial eradication had a lower Ki-67 LI compared with untreated *H. pylori*-infected mice and infected mice that received sulindac or YM022 (P < 0.05, respectively).

**Discussion**

The role of Cox-2 in promoting cancer is mainly attributed to PGE₂ production and its ability to promote cell proliferation,
migration, and angiogenesis and inhibit apoptosis (37). NSAIDs prevent some types of gastrointestinal cancer and other solid tumors in humans (37). In epidemiologic studies, the use of NSAIDs is generally associated with a lower risk of gastric cancer. However, a chemopreventive effect of NSAIDs on the development of gastric cancer among \textit{H. pylori}-infected individuals has not been conclusively shown in human clinical trials (36, 38). In patients that underwent \textit{H. pylori} eradication therapy, chronic use of celecoxib was associated with a higher regression rate of gastric precancerous intestinal metaplasia (36). However, in patients that had received \textit{H. pylori} eradication therapy, treatment with another selective Cox-2 inhibitor, rofecoxib, for 2 years did not reduce intestinal metaplasia (38). Considering that cancer chemoprevention by NSAIDs is modulated by both Cox-dependent and Cox-independent pathways (39), NSAIDs may have variable efficacy in their abilities to prevent gastric cancer.

Consistent with a role for Cox-2/PGE2 in carcinogenesis, double-transgenic mice expressing Cox-2 and \textit{Pges}-1 in the gastric mucosa overproduce PGE2 and develop gastric hyperplasia, metaplasia, and cancer in the glandular stomach (7). Because \textit{H. pylori} infection up-regulates Cox-2 expression and PGE2 production (5, 6), it is reasonable to hypothesize that suppression of PGE2 should prevent \textit{H. pylori}-associated gastric carcinogenesis. However, \textit{H. pylori}-associated gastritis and \textit{Tnf}-\alpha expression are more severe in \textit{Cox-1}-/- or Cox-2-/- mice (40) and in \textit{H. pylori}-infected mice receiving NSAIDs (41). Furthermore, Cox-2 inhibition enhances Th1 immune responses against \textit{H. pylori} (24, 25, 41). CD4/CD25 regulatory T-cell function dampens \textit{H. pylori}-associated gastritis in C56BL/6 mice (30). PGE2 enhances the inhibitory function of human regulatory T cells \textit{in vitro} (42). Therefore, regulatory T-cell function in the \textit{H. pylori}-infected mouse is likely to be compromised by reduced PGE2 production in sulindac-treated animals. Suppressed regulatory T-cell function may have also contributed to enhanced \textit{H. pylori}-associated inflammation in infected mice receiving sulindac alone, suggesting that PGE2 induced in response to \textit{H. pylori} infection plays a role in dampening gastric inflammation and reducing inflammation-mediated gastric carcinogenesis (2). The benefit of PGE2 suppression by NSAIDs in suppressing mediators of cancer progression, however, is not significant to counteract the adverse effect of Cox-2 inhibition associated with Th1-predominant inflammation caused by \textit{H. pylori} infection.

As described previously, \textit{H. pylori} infection promotes gastric carcinogenesis in INS-GAS male mice (8, 32). Although treatment with sulindac suppressed \textit{H. pylori}-induced gastric PGE2 levels, sulindac alone did not prevent gastric cancer in chronically infected INS-GAS mice. Moreover, sulindac exacerbated \textit{H. pylori}-induced gastritis and up-regulated expression of proinflammatory cytokines. In contrast, a combination of sulindac and antimicrobial therapy reduced gastric PGE2 levels and gastric inflammation and

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\caption{Cell proliferation in gastric mucosa: Ki-67 LI (A) and immunohistochemical staining of Ki-67 (B). Ki-67 LI were compared in corpus mucosa (A). \textit{H. pylori} infection resulted in significantly higher Ki-67 LI in mice (\textit{P} < 0.01). Sulindac or YM022 did not alter \textit{H. pylori}-associated increase of Ki-67 LI in INS-GAS mice. \textit{H. pylori}-infected mice receiving the combination of sulindac or YM022 and \textit{H. pylori} eradication had significantly lower Ki-67 LI compared with untreated infected mice (both \textit{P} < 0.05) or infected mice that received sulindac or YM022 (\textit{P} < 0.05, respectively). Proliferating cells were positively stained for Ki-67 (B). In \textit{Helicobacter}–uninfected INS-GAS mice, proliferating cells were restricted to the isthmus regions. Zone of proliferating cells expanded from isthmus regions to foveolar regions in the untreated \textit{H. pylori}–infected mice and infected mice that received sulindac or YM022. Proliferating cells were restricted to the isthmus regions irrespective of the hyperplastic foveolar glands in infected mice that received a combination of sulindac or YM022 and \textit{H. pylori} eradication. White column, \textit{H. pylori}–uninfected mice; black columns, \textit{H. pylori}–infected mice. Ki-67–positive cells were stained in brown. *, \textit{P} < 0.05; **, \textit{P} < 0.01 compared with uninfected untreated mice or comparison between indicated groups; #, \textit{P} < 0.05, compared with infected mice.}
\end{figure}
prevented the development of *H. pylori*-associated gastric cancer. These findings suggest that, in the presence of *H. pylori* infection, selected NSAIDs enhance Th1-predominant proinflammatory responses to *H. pylori* antigens (24) and may promote the development of inflammation-mediated gastric cancer in a susceptible host with chronic *H. pylori* infection (2). In previous studies, suppression of PGE2 by Cox-2 inhibitors did not alter Th2-associated II-4 expression; instead, the treatment up-regulated mRNA levels of Th1-associated II-12 and Tnf-z in gastric tissue of *H. pylori*-infected humans (24, 25). Sulindac treatment up-regulated gastric Ifn-γ and Tnf-z as well as II-4 mRNA in *H. pylori*-infected mice. Sulindac-mediated upregulation of Th1 proinflammatory cytokines positively correlated with the severity of *H. pylori*-associated gastric lesions in infected mice. Sulindac up-regulated gastric Ifn-γ and Tnf-z mRNA levels in *H. pylori*-uninfected INS-GAS mice, although gastric lesions and gastric cancer risk were reduced. Sulindac also up-regulated Cox-1 and Cox-2 mRNA levels in *H. pylori*-infected INS-GAS mice despite that it reduced gastric PGE2 levels as a result of its known Cox-inhibitory effects (43). Sulindac is a prodrug that is converted to sulfone and sulindac sulfide, and the sulfide form has been shown to activate peroxisome proliferator-activated receptor-γ and induce Cox-2 expression (44). The significance of up-regulated mRNA expression of Cox-1 and Cox-2 in *H. pylori*-infected mice that received sulindac is not clear and needs further study. The combination of sulindac and *H. pylori* eradication reduced the *H. pylori*-specific IgG2a titers, which were not significantly altered by sulindac treatment.

Sulindac prevented gastrin-driven carcinogenesis in *H. pylori*-uninfected male mice despite upregulation of proinflammatory cytokines. Moreover, the combination of sulindac and antimicrobial therapy was more effective in preventing gastric lesions, reducing cell proliferation in gastric mucosa and incidence of gastric cancer in *H. pylori*-uninfected mice whose stomachs are known to be colonized with enteric flora (32). Normal microbiota colonize all regions of the bowel, including the upper gastrointestinal tract of mice (45). Antimicrobial therapy with omeprazole, metronidazole, and clarithromycin resulted in dynamic changes of the gastric microbiota in humans (46). These findings in humans and mice suggest that gastrin flora other than *H. pylori* contribute to gastric inflammation and cancer (2).

Chemoprevention of *Helicobacter*-associated gastric cancer in INS-GAS mice was observed previously in long-term (6 months) suppression of CCK2/gastrin signaling by treatment with YF476, which was initiated during the acute stage of *Helicobacter* infection (27). We observed that the CCK2/gastrin receptor antagonist, YM022, alone or in combination with antimicrobial therapy reduced gastric cancer risk in *H. pylori*-uninfected INS-GAS mice. However, the chemopreventive effect of YM022 alone or in combination with antimicrobial therapy was not observed in *H. pylori*-infected INS-GAS mice when the 6-week treatment was initiated during the chronic stage of *H. pylori* infection. YM022 blockade of gastrin signaling (27) was not sufficient to counteract YM022-associated gastric pH elevation, which may facilitate overgrowth of certain gastric flora following *H. pylori* eradication therapy (32). These differing results in our current study and the previous report using YF476 might reflect intrinsic differences in the two antagonists but were more likely due to the different treatment schedules in these two studies. The data, however, are consistent with gastrin playing an important role early, but not later, in the multistep progression to gastric cancer (11).

Gastric proinflammatory cytokines and cell proliferation markers were down-regulated using the combination of YM022 and *H. pylori* eradication in *H. pylori*-infected INS-GAS mice, suggesting that this regimen has chemopreventive potential for gastric cancer. Regression of preneoplastic gastric intestinal metaplasia following *H. pylori* eradication is positively correlated with the interval that patients have been free of *H. pylori* infection (47). This suggests that regression of *H. pylori*-associated gastric lesions and gastric cancer risk may not be observed during the first few years after treatment (48). A longer period of follow-up may be necessary to examine the chemopreventive effect of *Helicobacter*-associated gastric cancer in INS-GAS mice using a combination of YM022 and *H. pylori* eradication.

Our data show that NSAIDs are an effective treatment modality in preventing of *H. pylori*-associated gastric cancer provided that *H. pylori* is eradicated before NSAIDs treatment is initiated. This study highlights the possible adverse effect of selected NSAIDs in promoting *H. pylori*-associated gastritis and cancer progression in susceptible hosts despite that PGE2 suppression by NSAIDs has chemopreventive properties in treatment of colon cancer and other solid tumors (19, 20, 37). A probable mechanism by which NSAIDs exacerbates *H. pylori*-associated gastritis and gastric cancer is via an enhanced proinflammatory Th1 immune response. Our study is consistent with the Maastricht III Consensus Report, which recommends *H. pylori* eradication for the prevention of peptic ulcer and gastric cancer, especially in patients with long-term NSAID usage (49).

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Acknowledgments**

Received 10/21/08; revised 7/27/09; accepted 8/20/09; published OnlineFirst 10/13/09.

Grant support: NIH grants R01AI07750, P01CA26571, P01ES02019 (J.G. Fox), and R01CA093405-07A1 (T.C. Wang and J.G. Fox).

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We thank Drs. Keiji Miyata and Hidenobu Yuki (Astellas Pharma, previously Yamanouchi Pharmaceutical) for providing YM022, Kristen Clapp and Juri Miyamae for technical assistance, and Kathy Corrimer for histology expertise.

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Cancer Research


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Combination of Sulindac and Antimicrobial Eradication of Helicobacter pylori Prevents Progression of Gastric Cancer in Hypergastrinemic INS-GAS Mice

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