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Hyperplastic gastric tumors induced by activated macrophages in COX-2/mPGES-1 transgenic mice

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Cyclooxygenase-2 (COX-2), the rate-limiting enzyme for prostanoid biosynthesis, plays a key role in gastrointestinal carcinogenesis. Among various prostanoids, prostaglandin E_2 (PGE_2) appears to be most responsible for cancer development. To investigate the role of PGE_2 in gastric tumorigenesis, we constructed transgenic mice simultaneously expressing COX-2 and microsomal prostaglandin E synthase (mPGES)-1 in the gastric epithelial cells. The transgenic mice developed metaplasia, hyperplasia and tumorous growths in the glandular stomach with heavy macrophage infiltrations. Although gastric bacterial counts in the transgenic mice were within the normal range, treatment with antibiotics significantly suppressed activation of the macrophages and tumorous hyperplasia. Importantly, the antibiotics treatment did not affect the macrophage accumulation. Notably, treatment of the transgenic mice with lipopolysaccharides induced proinflammatory cytokines through Toll-like receptor 4 in the gastric epithelial cells. These results indicate that an increased level of PGE_2 enhances macrophage infiltration, and that they are activated through epithelial cells by the gastric flora, resulting in gastric metaplasia and tumorous growth. Furthermore, *Helicobacter* infection upregulated epithelial PGE_2 production, suggesting that the COX-2/mPGES-1 pathway contributes to the *Helicobacter*-associated gastric tumorigenesis.

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Introduction

Regular use of nonsteroidal anti-inflammatory drugs (NSAIDs) is associated with a reduced risk of cancer development in the gastrointestinal tract (Thun *et al*, 1993; Farrow *et al*, 1998). The best-known targets of NSAIDs are cyclooxygenase (COX)-1 and COX-2 isoenzymes for prostaglandin $(PG)H_2$ synthesis. It has been established that inhibition of COX-2 is an effective chemoprevention strategy for colorectal cancer (Taketo, 1998a, b). Using $Apc^{\Delta 716}$ mice, a model for familial adenomatous polyposis, we have demonstrated that COX-2 plays a key role in the intestinal polyposis (Oshima *et al*, 1996, 2001). Accumulating evidence shows that COX-2 expression is also induced in the gastric cancer tissues (Ristimaki *et al*, 1997; Soydan *et al*, 1997). Gastric cancer is the second most common cancer in the world (Correa, 2003), and *Helicobacter pylori* infection is listed as a 'class I carcinogen' (IARC, 1994). In the *H. pylori*-associated gastritis and adenocarcinoma, COX-2 is induced significantly, whereas its level is decreased dramatically upon *H. pylori* eradication (Fu *et al*, 1999; Sung *et al*, 2000). Furthermore, treatment of the *H. pylori*-infected mice with NSAIDs or COX-2 inhibitors suppresses the gastric hyperplasia (Kim *et al*, 2001; Xiao *et al*, 2001). These results suggest that COX-2 plays a pivotal role in the *H. pylori*-associated gastric tumorigenesis.

PGE_2 is one of the key prostanoids responsible for tumorigenesis. In the $Apc^{\Delta 716}$ polyps, PGE_2 signaling through its receptor EP2 is responsible for angiogenesis and construction of the basement membrane (Sonoshita *et al*, 2001; Seno *et al*, 2002). Other effects of PGE_2 on tumorigenesis have also been suggested, such as increased cell survival and motility (Sheng *et al*, 1998, 2001), inhibition of host immune responses (Huang *et al*, 1998) and activation of the EGF receptor signal (Pai *et al*, 2002). Moreover, it has been demonstrated that a long-term treatment of rats with 16,16-dimethyl PGE_2 increases mucosal thickness in the stomach, accompanied by elevated numbers of mucosal cells (Reinhart *et al*, 1983). However, the biological mechanism(s) of how PGE_2 is involved in tumorigenesis remains to be elucidated.

It has been reported that microsomal PGE synthase (mPGES)-1, one of the PGE_2 synthases, is co-localized and functionally coupled with COX-2 (Murakami *et al*, 2000; Lazarus *et al*, 2002). Furthermore, induction of mPGES-1 is also accompanied by COX-2 induction in colon cancer tissues (Yoshimatsu *et al*, 2001). Therefore, it is possible that a simultaneous induction of both COX-2 and mPGES-1 stimulates tumorigenesis synergistically.

To investigate the precise mechanism of the PGE_2 action on the gastric epithelial cell growth, we have constructed transgenic mice simultaneously expressing both COX-2 and mPGES-1 in the gastric mucosa. Here we demonstrate that the transgenic mice develop hyperplastic gastric tumors, and that the increased levels of PGE_2 cause these phenotypes through recruitment of mucosal macrophages and their stimulation mediated through epithelial Toll-like receptor (TLR) 4 activated by the indigenous bacterial flora.

Results

The transgenic mice express COX-2 and mPGES-1 in glandular stomach

To investigate the effects of PGE_2 on the gastric mucosa, we constructed transgenic mice that express both COX-2 and

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mPGES-1 by co-microinjection of two expression vectors (Figure 1A). We used cytokeratin 19 (K19) promoter to target the epithelial cells of gastric mucosa (Brembeck *et al*, 2001). Two transgenic lines, *K19-C2mE-2* and *K19-C2mE-8*, showed high expression levels of the transduced genes and essentially the same phenotypes (data not shown). Accordingly, we present the results with *K19-C2mE-8* (hereafter *K19-C2mE*) mice. The *K19-C2mE* mice expressed the transgene-encoded COX-2 and mPGES-1 in the forestomach and glandular stomach, whereas expression of the endogenous COX-2 was undetectable and mPGES-1 was weakly expressed in the whole digestive tract (Figure 1B). The PGE_2 levels in the glandular stomach of *K19-C2mE* mice were approximately twice as high as those in the wild-type mice (Figure 1C). These results indicate that forced expression of COX-2 and mPGES-1 efficiently stimulates PGE_2 biosynthesis in the gastric epithelial cells.

The K19-C2mE mice show aberrant differentiation of the gastric mucosa

In the transgenic mice, COX-2 and mPGES-1 were detected essentially in the surface epithelial cells of glandular stomach (Figure 2A). At 12 weeks of age, the *K19-C2mE* mice showed abnormal gastric histology of the glandular stomach, with elongated gastric pits. Furthermore, the mucous cell population was found expanded in the whole gland, as detected by *Helix pometia*-lectin staining (Figure 2B). The numerous

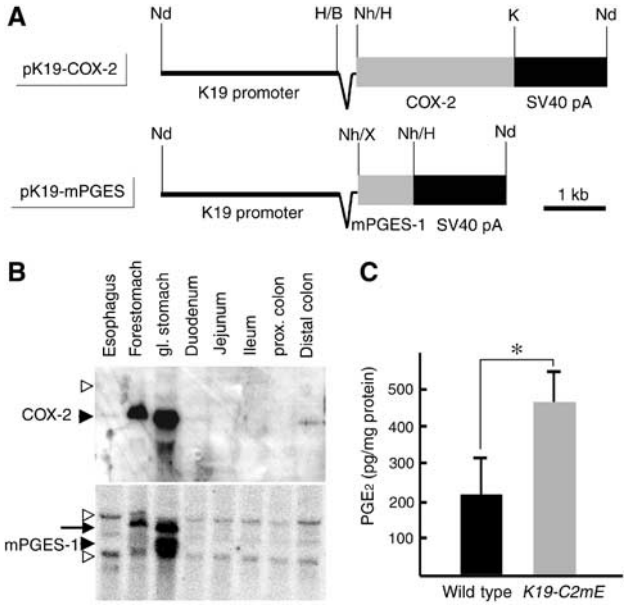

Figure 1 Expression of COX-2 and mPGES-1, and production of PGE_2 in *K19-C2mE* stomach. (**A**) Transgenic vectors for COX-2 and mPGES-1 expression, respectively. Synthetic chimeric intron is indicated as a wedge-shaped line between the K19 promoter and each cDNA. Fragments of cDNAs and SV40 pA cassettes are indicated as gray and black boxes, respectively. Nd, *Nde*I; H, *Hind*III; B, *Bam*HI; K, *Kpn*I; Nh, *Nhe*I; and X, *Xba*I. (**B**) Northern blots for COX-2 and mPGES-1 mRNAs in the *K19-C2mE* mouse digestive tract. Filled arrowheads indicate the mRNA sizes for transgenic genes, whereas open arrowheads indicate the sizes for endogenous genes. The arrow on the bottom panel indicates the residual COX-2 band because of the reprobing of the same filter as the top. (**C**) PGE_2 levels in the glandular stomach are presented as the mean $\pm$ s.d. $^*P<0.05$.

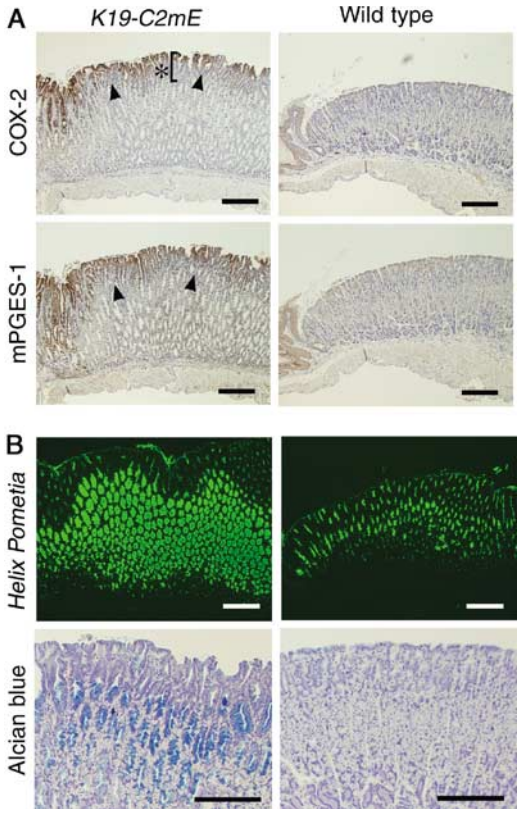

Figure 2 Histological analyses of the glandular stomach at 12 weeks of age, in the *K19-C2mE* mice (left) and wild type (right). (**A**) Immunohistochemistry for COX-2 (top) and mPGES-1 (bottom). Arrowheads indicate the positive staining on mucosal surface. Asterisk in the top left panel indicates elongated pit. (**B**) Mucous metaplasia in the gastric gland. *Helix pometia*-lectin staining (top) and Alcian blue staining (bottom). Bars in (A) and (B), 200 μm.

mucous cells in *K19-C2mE* mice contained acidic mucins stained with Alcian blue, which were not produced in the normal gastric mucosa. These results indicate that the increased level of PGE_2 disturbs normal differentiation of epithelial cells of the gastric mucosa.

K19-C2mE mice develop gastric hyperplasia and tumorous growths

We next determined the cell proliferation rate by bromodeoxyuridine (BrdU) incorporation. In the wild-type mice, proliferating cells were detected only in the neck of the gastric gland (Figure 3A). In the *K19-C2mE* mice, however, the BrdU-labeled cells were found in the gland bottom as well as in the neck. Moreover, the labeling index per gland in the *K19-C2mE* stomach was twice higher than that in the wild-type mice (Figure 3B). At 48 weeks of age, large tumorous growths were found in the proximal glandular stomach (Figure 3C). Histopathology of the tumors showed benign metaplastic hyperplasia consisting of Alcian blue-positive mucous cells and other types of differentiated epithelial cells. We confirmed expression of both COX-2 and mPGES-1 in the tumor epithelial cells by immunohistochemistry.

To examine whether the gastric tumorous hyperplasia is dependent on the COX-2 activity, we treated the mice with a COX-2 selective inhibitor NS-398. In the no-drug control mice, the gastric mucosal thickness at the proximal stomach

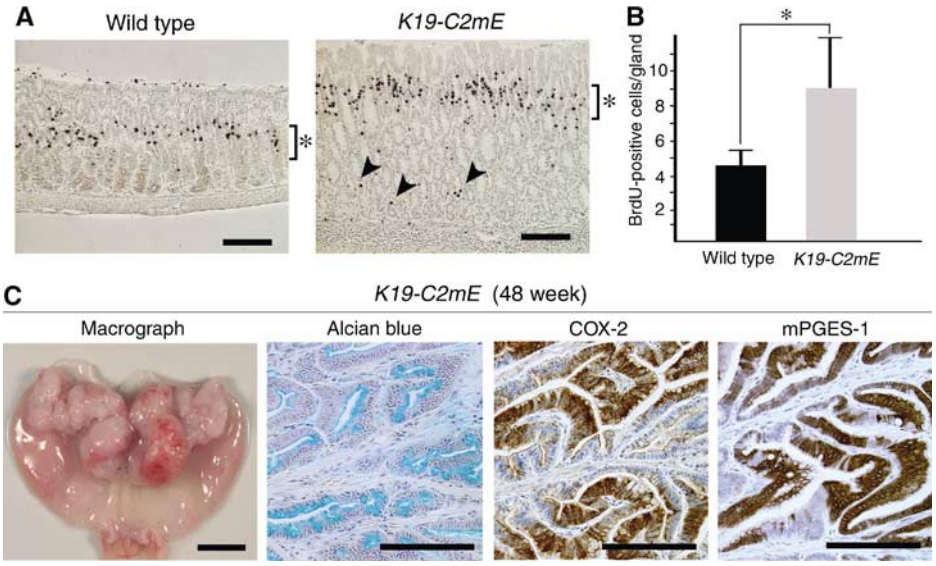

Figure 3 Hyperplasia and tumor development in the *K19-C2mE* glandular stomach. (**A**) BrdU staining after 1 h labeling at 12 weeks of age. Asterisks indicate BrdU-labeled cells in the gland neck. Arrowheads in the *K19-C2mE* stomach indicate BrdU-positive cells in the gland bottom. Bars, 500 μm. (**B**) BrdU labeling indices are presented as the mean ± s.d. $*P<0.05$. (**C**) Tumor development at 48 weeks. From left to right: macroscopic photograph, Alcian blue staining, immunostaining for COX-2 and mPGES-1. Bars in macrograph and histological sections, 5 mm and 200 μm, respectively.

increased gradually with age, to more than twice of that in the wild type by 20 weeks (Figure 4A and B). However, treatment of *K19-C2mE* mice with NS-398 for 4 weeks completely suppressed the gastric hypertrophy, reducing the mucosal thickness to that in the age-matched wild type. These results clearly indicate that the increased levels of PGE_2 are essential for the gastric pathology in *K19-C2mE* mice.

Bacterial infection triggers gastric hyperplasia in K19-C2mE mice

Numerous mononuclear cells were found infiltrating in the submucosa in *K19-C2mE* mice (Figure 4C). To determine whether gastric bacterial flora was responsible for these inflammatory responses and gastric hyperplasia, we treated *K19-C2mE* mice with antibiotics, streptomycin and cefoperazone for 3 weeks. The treatment eliminated gastric bacteria to undetectable levels (Figure 4D) and cleared the submucosal infiltration of mononuclear cells (Figure 4C). Furthermore, such a treatment decreased the mucosal thickness remarkably from 2.4 to 1.7 times of the wild type (Figure 4A and B). It is established that the same antibiotics with the same dosing protocol as in this study do not affect cell proliferation in the mouse gastric mucosa (Zavros *et al*, 2002a), excluding the possibility that the suppressive effect was caused directly by the antibiotics against the mucous cells.

We next determined gastric bacterial counts under aerobic, microaerophilic and anaerobic conditions, respectively. The mean bacterial counts of the *K19-C2mE* mice were higher than those in the wild type under all culture conditions. However, the bacterial counts were still within the normal range in nearly half of the transgenic mice (gray zones in Figure 4D), although all transgenic mice developed gastric hypertrophy regardless of their bacterial counts (data not shown). These results, taken together, indicate that host inflammatory responses in *K19-C2mE* mice can be triggered by the normal gastric flora and cause the gastric hyperplasia and hypertrophy. Increased bacterial counts in some transgenic mice are possibly caused by the weakened gastric acidity in the metaplastic epithelial cells (Figure 2B).

Lipopolysaccharide (LPS) stimulates gastric epithelial cells through TLR4

Upon close histological examination of the *K19-C2mE* transgenic mice, we could not find any signs of bacterial invasion or colonization in the gastric mucosa (data not shown). Accordingly, it was expected that epithelial cells, rather than stromal cells, were stimulated directly by the gastric bacteria. TLR2 and TLR4 recognize such bacterial pathogens as peptidoglycan (PGN) and LPS, respectively, and play important roles in innate immune responses (Kaisho and Akira, 2002). We found expression of both TLR2 and TLR4 mRNAs by RT–PCR in the total RNA from the whole glandular stomach (data not shown). Interestingly, we detected only TLR4 mRNA in the gastric mucosa excised by the laser microdissection (LMD) method, and in the primary culture of the gastric epithelial cells (Figure 5A). Consistent with the results, treatment of the gastric epithelial cells with PGN did not induce tumor necrosis factor (TNF)-α significantly (Figure 5B). In contrast, LPS stimulation induced TNF-α in a dose-dependent manner, although the expression level was ~1000-fold lower than that detected in the LPS-stimulated mouse monocyte line, RAW264 (Figure 5C). We also detected expression of interleukin (IL)-1β in the LPS-stimulated epithelial cells by RT–PCR (data not shown). Induction of TNF-α was efficiently suppressed by treatment with TLR4-blocking antibody in both the gastric epithelia and RAW264, although the antibody itself could induce TNF-α slightly (Figure 5C). Interestingly, the levels of TLR4 expression and TNF-α induction were essentially the same in *K19-C2mE* and wild-type gastric epithelial cells. Therefore, it is possible that normal gastric flora stimulates the epithelial cells through

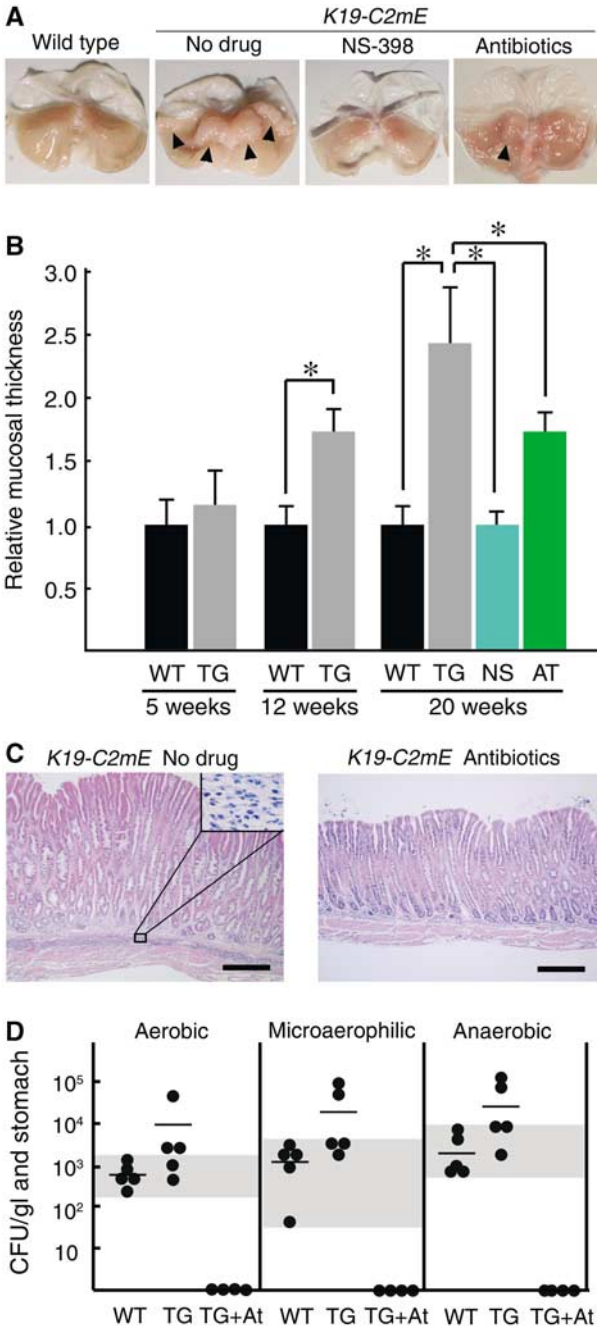

Figure 4 Suppression of gastric hyperplasia either by treatment with a COX-2 inhibitor (NS-398) or antibiotics. (**A**) Macroscopic photographs of the stomach at 20 weeks of age of the wild-type (left) and *K19-C2mE* (right) mice, with respective treatments as indicated. Arrowheads in 'no-drug' indicate the hypertrophic lesion. In the antibiotics-treated stomach, a mild hypertrophic lesion is still found (arrowhead). (**B**) Relative mucosal thickness at 5, 12 and 20 weeks of age is presented as the mean ± s.d. (i.e., the mean thickness divided by that of the wild type). WT, wild type; TG, nontreated *K19-C2mE*; NS and AT, *K19-C2mE* treated with NS-398 and antibiotics, respectively. $^*P<0.05$. (**C**) Histopathology of the glandular stomach in the untreated (left) and antibiotics-treated (right) *K19-C2mE* mice, respectively (H&E staining). Inset in the left panel shows a higher magnification of the mononuclear cell-infiltrated submucosa. Bars, 300 μm. (**D**) Bacterial counts in the glandular stomach at 20 weeks of age. CFUs per mouse glandular stomach are indicated as closed circles. Horizontal bars indicate the mean CFU. Culture conditions are shown on top. WT, wild type; TG, *K19-C2mE*; TG + At, *K19-C2mE* with antibiotics. Gray horizontal zones indicate the ranges of bacterial counts in wild-type mice.

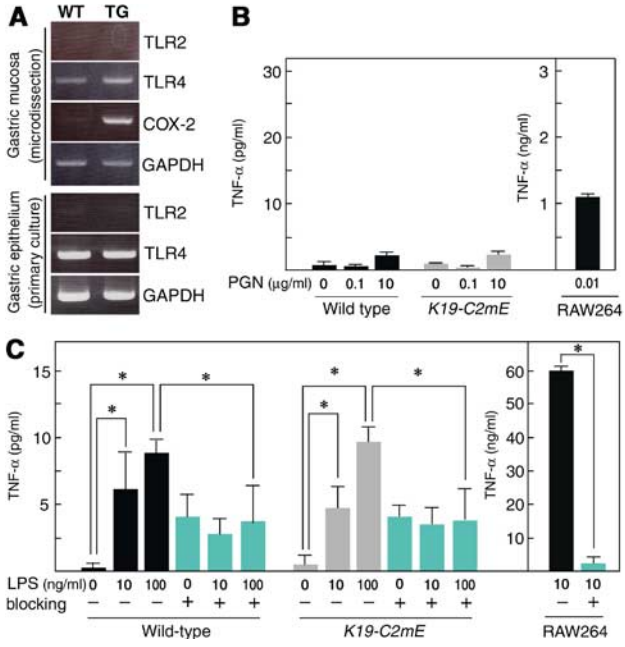

Figure 5 Stimulations of TLR on the gastric epithelial cells with bacterial PGN or LPS. (**A**) RT–PCR for TLR2 and TLR4 mRNAs using total RNA from gastric mucosa sampled by LMD (top) and primary culture of the epithelial cells (bottom), respectively. COX-2 in LMD-sample RT–PCR was used as positive control. GAPDH was used as endogenous controls. WT, wild type; TG, *K19-C2mE*. Gastric epithelial cells were treated with PGN (**B**) and LPS (**C**) for 20 h. Concentrations of TNF-α in the supernatants are presented as the mean ± s.d. RAW264 mouse monocyte cells were used for positive control. For TLR4 blocking, anti-TLR4 antibody was added at 10 μg/ml (blue bars). $^*P<0.05$.

TLR4 weakly but continuously both in the wild-type and transgenic mice, and that the TLR4 activation in the epithelial cells indirectly induces inflammatory responses in the macrophage-accumulated *K19-C2mE* stomach. We did not detect any TNF-α or IL-1β in the epithelial cells by immunohistochemistry, possibly because of low expression levels.

Inflammatory cytokines, chemokines and growth factors are induced in K19-C2mE mice

To characterize the tissue microenvironment that caused gastric hyperplasia and hypertrophy, we next determined mRNA levels for various proinflammatory cytokines, chemokines and growth factors in the whole glandular stomach. Among them, only TNF-α and CXCL14 (BRAK) were increased significantly at 3 weeks of age (Figure 6A). At 20 weeks, the mRNA levels for proinflammatory cytokines IL-1β and IL-6 and chemokine MIP-2 (murine homolog of CXCL2/3) were increased significantly in addition to TNF-α and CXCL14 (Figure 6B). These results were consistent with the inflammatory histopathology (Figure 4C). Expression of these cytokines and chemokines was found in the stroma by immunohistochemistry (Figures 7C and 8A; data not shown). The levels of granulocyte-macrophage colony-stimulating factor (GM-CSF), hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF) were also increased significantly at 20 weeks of age.

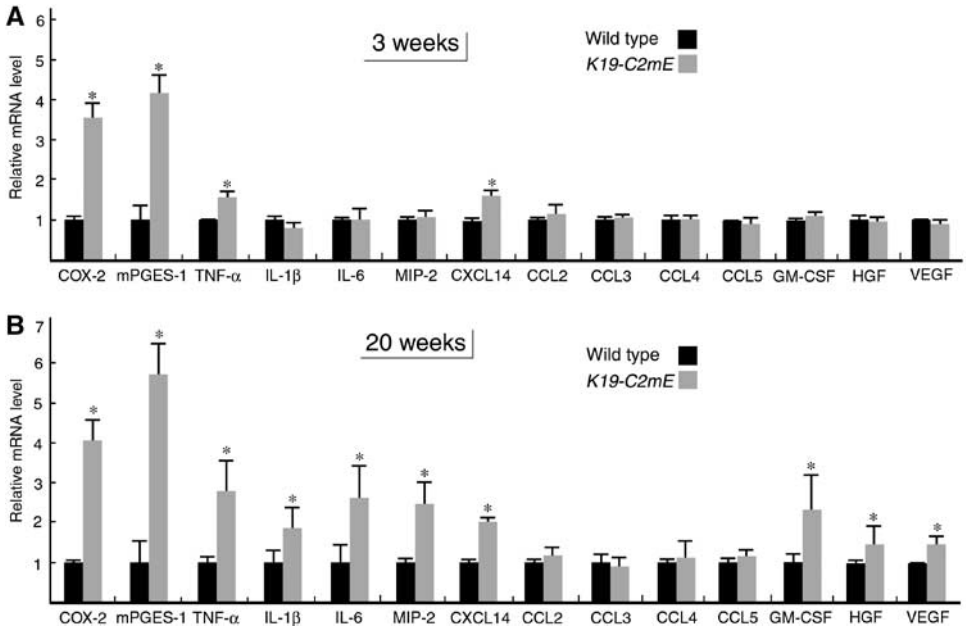

Figure 6 mRNA levels for cytokines, chemokines and growth factors in the glandular stomach at 3 weeks (**A**) and 20 weeks (**B**) of age. Relative mRNA levels in *K19-C2mE* mice compared with those in the wild type are presented as the mean ± s.d. $*P<0.05$ versus wild type.

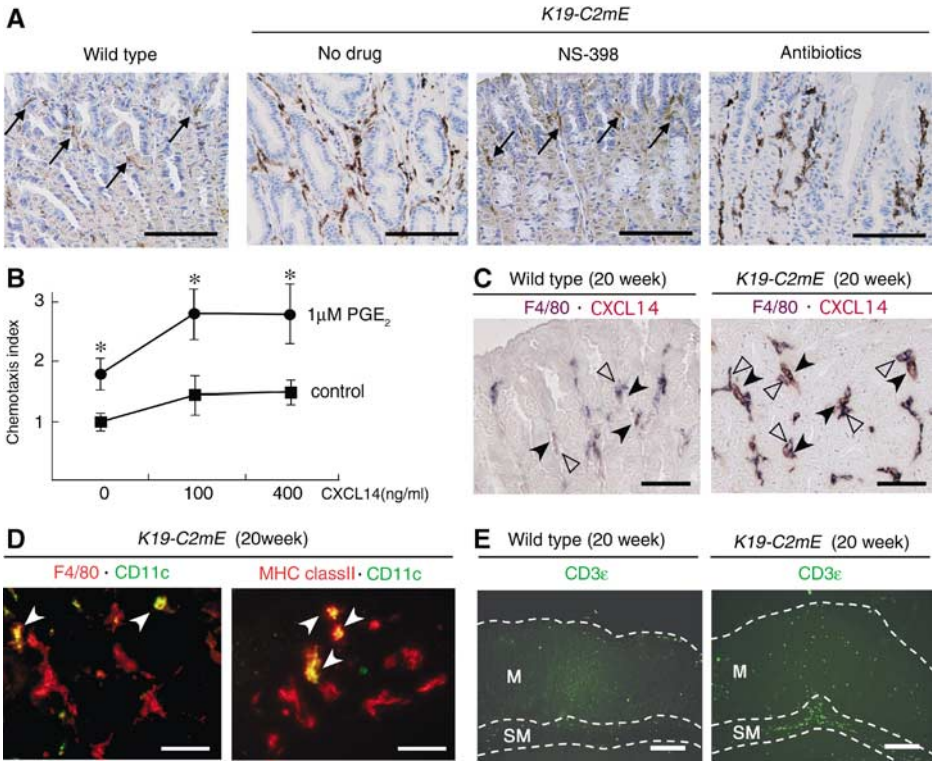

Figure 7 Macrophage infiltration in the glandular stomach of *K19-C2mE* mice. (**A**) Immunostaining for macrophage marker F4/80 at 20 weeks of age. Arrows in wild type and NS-398-treated *K19-C2mE* indicate scarce macrophages. Numerous macrophages are found infiltrating in the 'no-drug' control *K19-C2mE* mucosa. Note that a similar macrophage infiltration is still found even after the antibiotics treatment. Bars, 200 μm. (**B**) Relative chemotaxis index of RAW264 cells to CXCL14. Cells were cultured in the presence (circles) or absence (squares) of 1 μM PGE_2 for 20 h prior to migration assays. $*P<0.05$ versus untreated control cells. (**C**) Double immunostaining for F4/80 (purple) and CXCL14 (brown) in the wild type (left) and *K19-C2mE* (right) at 20 weeks of age. Closed arrowheads indicate the CXCL14-expressing stromal cells, whereas open arrowheads show F4/80-positive macrophages. Bars, 100 μm. (**D**) Merged images of the double fluorescence immunostaining for F4/80 (red) and CD11c (green) (left), and MHC class II (red) and CD11c (green) (right). White arrowheads indicate double-positive cells (yellow). Bars, 50 μm. (**E**) Fluorescence immunostaining for CD3ε (green). Boundaries for mucosa (lamina propria) (M) and submucosa (SM) are drawn with a white dashed line. Bars, 200 μm.

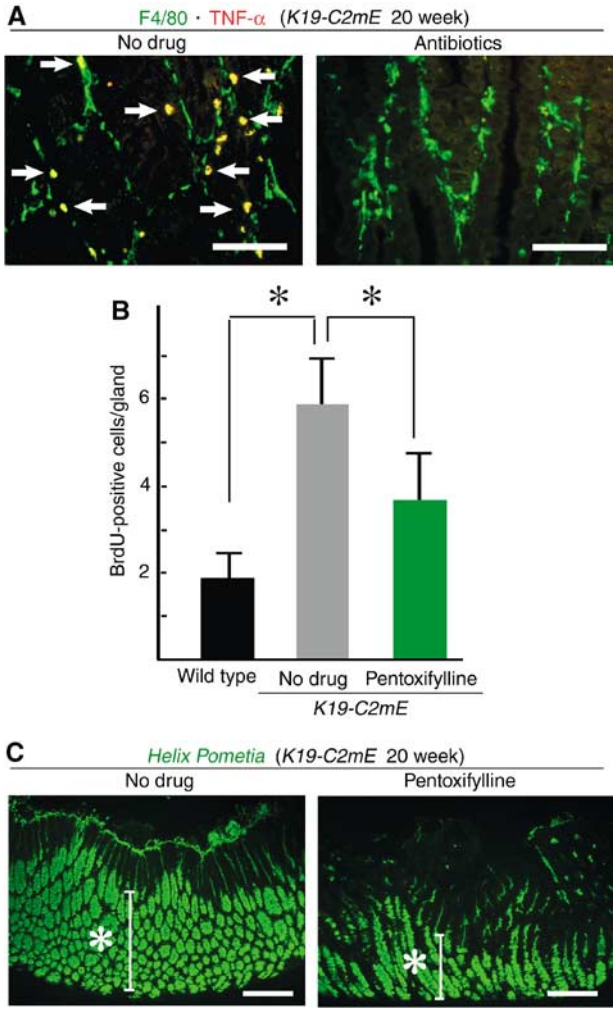

Figure 8 Macrophage TNF-α in gastric hyperplasia. (**A**) Merged images of double immunostaining for F4/80 (green) and TNF-α (red) in *K19-C2mE* mice at 20 weeks of age. No-drug control (left) and antibiotics-treated (right). White arrows indicate double-positive cells (yellow). Bars, 200 μm. (**B**) BrdU labeling indices in the glandular stomach of the wild type, no-drug *K19-C2mE* and pentoxifylline-treated *K19-C2mE* mice are presented as the mean ± s.d. $^*P<0.05$. (**C**) *Helix pometia*-lectin staining in a *K19-C2mE* mouse at 20 weeks of age. No-drug control (left) and pentoxifylline-treated (right). Bars, 200 μm.

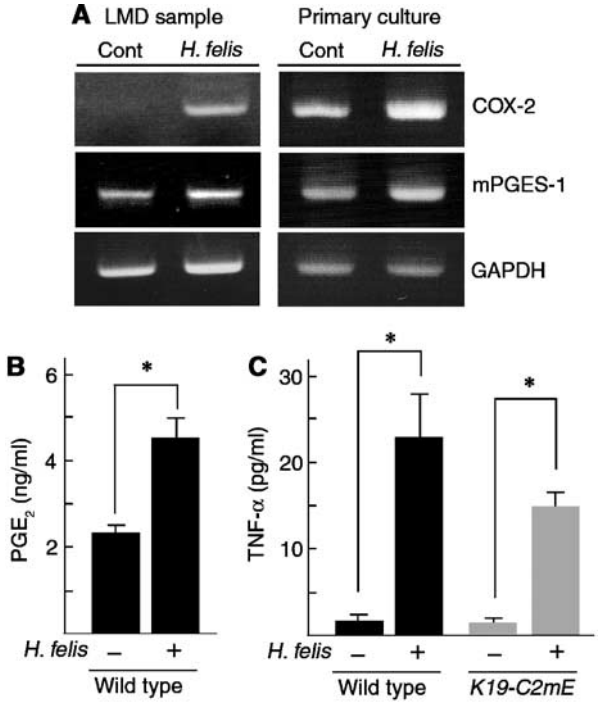

Figure 9 Infection of *H. felis* to gastric epithelial cells. (**A**) RT–PCR for COX-2 and mPGES-1 mRNA in surface epithelial cells of the glandular stomach of *H. felis*-infected and control mice sampled by LMD (left) and those of *H. felis*-infected and control primary culture of gastric epithelial cells (right). GAPDH was used for endogenous control. Note that the expression of COX-2 and mPGES-1 is increased by *H. felis* infection both *in vivo* and *in vitro*. (**B**) PGE_2 levels in the culture medium of *H. felis*-infected and control gastric epithelial cells are presented as the mean ± s.d. $^*P<0.05$. (**C**) Gastric epithelial cells of wild-type and *K19-C2mE* were infected with *H. felis*. Concentrations of induced TNF-α in supernatants are presented as the mean ± s.d. $^*P<0.05$ versus uninfected cells.

Increased PGE_2 levels help recruit macrophages in the glandular stomach

To investigate whether mucosal macrophages were involved in inflammatory responses in *K19-C2mE*, we next examined the glandular stomach immunohistochemically with a macrophage marker F4/80 (Figure 7A). In the wild-type mice, tissue macrophages were sparsely scattered in the mucosal stroma. In contrast, a heavy macrophage infiltration to mucosal stroma was found in *K19-C2mE* mice. Treatment of *K19-C2mE* mice with NS-398 effectively suppressed the macrophage infiltration to the wild-type level. However, numerous macrophages were still found in the stomach of the antibiotics-treated mice, despite the fact that the bacterial infection was eliminated (Figure 4D). Accordingly, the increased PGE_2 level is the likely primary cause of the enhanced macrophage infiltration, regardless of the epithelial responses to gastric bacteria.

CXCL14 is a homeostatic chemokine that plays a role in monocyte recruitment to normal tissues (Muller, 2001), and cultured monocytes become more responsive to CXCL14 in the presence of PGE_2 (Kurth *et al*, 2001). We confirmed that treatment of RAW264 cells with PGE_2 significantly enhanced chemotaxis to CXCL14 (Figure 7B). Consistent with the RT–PCR results, many stromal cells of the *K19-C2mE* glandular stomach showed strong staining for CXCL14, whereas only a small number of cells were stained weakly in the wild-type stomach (Figure 7C). Numerous CXCL14-positive stromal cells were also found in the antibiotics-treated stomach (data not shown), suggesting that production of CXCL14 was stimulated by PGE_2, rather than bacterial infection. Importantly, we found F4/80-positive macrophages adjacent to the CXCL14-producing cells, which suggests that CXCL14 helps recruit macrophages in the *K19-C2mE* gastric mucosa. Notably, treatment of RAW264 cells with PGE_2 enhanced cell migration even in the absence of CXCL14 (Figure 7B). As RAW264 does not express CXCL14 (data not shown), other factors may also be involved in PGE_2-stimulated macrophage infiltration. Although CCL2 (monocyte chemotactic protein (MCP)-1), CCL3 (MIP-1α), CCL4 (MIP-1β) and CCL5 (RANTES) can also recruit monocytes or macrophages (Homey *et al*, 2002), these chemokines are unlikely to have caused macrophage infiltration in *K19-C2mE* mice because their expression levels were not increased (Figure 6B).

We also characterized other types of infiltrating cells in the *K19-C2mE* gastric mucosa. When double stained with an antibody for dendritic cell marker CD11c, together with that for F4/80 (Figure 7D), only a small subpopulation of the F4/80-positive cells were stained for CD11c. Interestingly, there were numerous cells expressing the MHC class II antigen, but only a few of them were $CD11c^+$ dendritic cells (Figure 7D), suggesting that the majority of the F4/80-positive cells consisted of activated macrophages. Consistent with the histopathology (Figure 4C), most T cells visualized by CD3ε were found in the submucosa of *K19-C2mE* mice (Figure 7E). In the gastric mucosa (lamina propria), however, the number of T cells was slightly higher in *K19-C2mE* mice than in the wild type. Moreover, we found $B220^+$ B cells and $Gr\text{-}1^+$ granulocytes mainly in the submucosa, and only rarely in the mucosa (data not shown).

Suppression of proinflammatory cytokines reduces proliferation of the gastric epithelial cells

We next determined the cell type(s) that expresses TNF-α in the mucosal stroma by fluorescence double immunostaining with F4/80 antibody. Abundant TNF-α was detected primarily in the mucous macrophages in *K19-C2mE* mice (Figure 8A). In contrast, macrophages in the antibiotics-treated mice seldom produced TNF-α, indicating that gastric bacterial flora indirectly induced TNF-α in the infiltrating macrophages.

TNF-α is a key mediator of inflammation, and also plays important roles in the early stages of tumorigenesis (Moore *et al*, 1999; Knight *et al*, 2000). In addition, activated macrophages produce other proinflammatory cytokines, such as IL-1 and IL-6 (Cavaillon, 1994), levels of which were elevated also in *K19-C2mE* mice (Figure 6). To investigate the effects of these cytokines on the gastric hyperplasia, we treated *K19-C2mE* mice with pentoxifylline, which inhibits production of TNF-α, IL-1 and IL-6 (Voisin *et al*, 1998). Only after a short-term treatment (5 days), pentoxifylline substantially reduced the BrdU-labeling index of the epithelial cells in *K19-C2mE* mice compared with that in the untreated controls (Figure 8B). The number of metaplastic mucous cells in the glandular stomach was also decreased in the pentoxifylline-treated mice (Figure 8C). Therefore, the proinflammatory cytokines produced by activated macrophages should significantly contribute to the gastric metaplasia and hyperplasia in the transgenic mice.

Helicobacter infection induces PGE_2 production in gastric epithelial cells

It has been reported that infection of mice with *H. pylori* or its close relative *H. felis* results in mucous metaplasia and hyperplasia of the glandular stomach (Lee *et al*, 1990, 1997; Fox *et al*, 1996). Therefore, we examined whether the COX-2/mPGES-1 pathway is involved in the gastric phenotypes of *Helicobacter* infection models, using frozen infected tissues and primary culture of the gastric epithelial cells. In the surface epithelial cells of mouse glandular stomach, *in vivo* infection of *H. felis* induced COX-2 mRNA, while the mPGES-1 mRNA increased only slightly (Figure 9A). In the primary gastric epithelial cells, *H. felis* infection also increased both COX-2 and mPGES-1 (Figure 9A). Consistently, the PGE_2 level in the primary culture medium of *H. felis*-infected cells was two times higher than that of the control (Figure 9B). Moreover, *H. felis* infection stimulated expression of TNF-α in both wild-type and *K19-C2mE* epithelial cells at the same level (Figure 9C). These results are consistent with a recent report that COX-2 is induced in the mouse gastric epithelial cells *in vivo* by *H. pylori* infection (Jüttner *et al*, 2003). On the other hand, it has been reported that *H. pylori* LPS stimulates TLR4 of gastric pit cells (Kawahara *et al*, 2001), whereas *H. pylori* LPS and flagellin stimulate TLR2 and TLR5 of gastric cancer cells, respectively (Smith *et al*, 2003). Moreover, *H. pylori* proteins, membrane protein 1 and urease B, can induce TNF-α (Suganuma *et al*, 2001). While multiple mechanisms may be involved, our present results establish that *H. felis* infection induces TNF-α in the normal gastric epithelial cells. Therefore, activation of the COX-2/mPGES-1 pathway, together with induction of TNF-α in the gastric epithelial cells, contributes to the gastric phenotypes by the *Helicobacter* infection.

Discussion

Increased PGE_2 level in the gastric mucosa enhances recruitment of tissue macrophages

It is widely accepted that a functional relationship exists between inflammation and tumor cell growth (Balkwill and Mantovani, 2001; Coussens and Werb, 2002). It is estimated that over 15% of cancer in the world is caused by infections that often induce persistent chronic inflammations in the hosts (Kuper *et al*, 2000). Activated macrophages in the inflammatory sites may supply cytokines and growth factors to the tumor epithelial cells. These tumor-associated macrophages (TAM) are derived from monocytes that have been recruited largely by chemokine MCPs (Balkwill and Mantovani, 2001; Coussens and Werb, 2002). Here we have demonstrated that an increased PGE_2 level, which is characteristic of inflammatory responses, plays an important role in macrophage infiltration into the gastric stroma. As enhanced macrophage infiltration was suppressed by COX-2 inhibition, but not by elimination of the gastric bacterial flora, we conclude that increased levels of PGE_2 play a crucial role in recruiting monocytes into the gastric mucosa of *K19-C2mE* mice.

It has been demonstrated that circulating monocytes migrate to the normal (i.e. un-inflamed) tissues guided by constitutively expressed chemokine CXCL14 (Kurth *et al*, 2001; Muller, 2001). Furthermore, PGE_2 is shown to upregulate dramatically the monocyte responsiveness to CXCL14 (Kurth *et al*, 2001). Considering the facts that CXCL14 expression is increased in *K19-C2mE* mice (Figure 6 and 7C) and that macrophages are found adjacent to CXCL14-expressing cells (Figure 7C), it is conceivable that CXCL14 is one of the key factors for mucosal macrophage infiltration. As PGE_2 treatment stimulates migration of RAW264 cells in the absence of CXCL14 (Figure 7B), additional factors may also be involved in the PGE_2-dependent monocyte recruitment.

Macrophage activation is responsible for gastric hyperplasia

It has been well documented that infections of *H. pylori* and its close relative *H. felis* cause chronic inflammation and hyperplasia in the mouse glandular stomach (Lee *et al*, 1990, 1997; Fox *et al*, 1996). Infection of mice with other bacterial species, *H. heilmannii* or *Acinetobacter lwoffi*, can induce

gastric metaplasia and hyperplasia (Peterson *et al*, 2001; Zavros *et al*, 2002a). Importantly, such gastric mucosal hypertrophy/nodular hyperplasia by *H. felis* was not induced in T-cell-deficient mice, RAG-1$^{-/-}$ or TCR$\beta\delta^{-/-}$ mutants (Roth *et al*, 1999). These results indicate that host immune responses are essential for the gastric pathology. In the present study, we have provided direct evidence that host inflammatory responses to the normal gastric flora can cause similar phenotypes. Moreover, we have demonstrated that bacterial LPS stimulates TLR4 on the gastric epithelial cells to induce proinflammatory cytokines activating mucosal macrophages of *K19-C2mE* mice. Accordingly, it is conceivable that PGE_2-dependent heavy macrophage infiltrations together with cytokine signals from the epithelial cells, are important for inflammatory responses and gastric hypertrophy triggered by gastric bacteria.

Proinflammatory cytokines are key molecules in gastric hyperplasia

It has been reported that TNF-α plays a key role in the early stages of carcinogen-induced tumorigenesis in the skin and liver (Moore *et al*, 1999; Knight *et al*, 2000). Inhibition of proinflammatory cytokine production by pentoxifylline decreased the epithelial cell proliferation rate in *K19-C2mE* mice. This effect was found in only 5 days, when immune cells were still present in the submucosa (data not shown). Thus, it is possible that macrophage-derived proinflammatory cytokines participate in the gastric tumorigenesis. It remains to be determined which particular molecule is most responsible for the gastric hyperplastic tumorigenesis.

It is also possible that upregulation of HGF and VEGF in the *K19-C2mE* glandular stomach is mediated by macrophage-derived proinflammatory cytokines. HGF has been implicated in gastric tumorigenesis because of its upregulation in gastric cancer (Konturek *et al*, 2001), whereas VEGF is a key angiogenic factor in tumor tissues (Seno *et al*, 2002). Thus, these growth factors induced by proinflammatory cytokines can contribute to the gastric phenotypes in *K19-C2mE* mice.

H. pylori-associated gastric tumorigenesis may be suppressed more efficiently by COX-2 inhibition coupled with antibiotics

A chemoprevention trial showed that antimicrobial therapy against *H. pylori* improved the regression of gastric cancer precursor lesions (Correa *et al*, 2000). Moreover, accumulating evidence suggests that COX-2 plays a key role in *H. pylori*-associated gastric pathology (Fu *et al*, 1999; Sung *et al*, 2000). Here, we have demonstrated two important steps in gastric tumorigenesis that are triggered by increased levels of PGE_2. First, PGE_2 induces macrophage infiltration in the stomach, which can be stimulated by gastric infectious agents activating epithelial TLR4. Second, macrophage activation accelerates gastric epithelial growth through upregulation of proinflammatory cytokines and growth factors. It has been well established that expression of COX-2 as well as proinflammatory cytokines, such as TNF-α, IL-1β, IL-6 and IL-8 (CXCL8), is induced in *H. pylori*-associated gastric cancer (Crabtree *et al*, 1991; Noach *et al*, 1994). We have demonstrated that *H. felis* infection induces expression of both COX-2 and mPGES-1 in the gastric epithelial cells, suggesting that mucosal macrophages are also recruited in the *Helicobacter*-infected stomach. In addition, *H. felis* infection induced epithelial expression of TNF-α, although the molecular mechanism for TNF-α induction remains to be investigated further. Accordingly, this two-step mechanism triggered by PGE_2 (i.e., macrophage infiltration followed by their activation) can also explain the *H. pylori*-associated gastric tumorigenesis. Therefore, COX-2 inhibition combined with antibiotics administration can be an effective chemopreventive strategy for *H. pylori*-associated gastric cancer.

In conclusion, inhibition of the PGE_2 production should be an effective strategy in suppressing macrophage infiltration and hyperplastic cell growth in the gastric mucosa through cytokine networks.

Materials and methods

Transgenic mice

A 2.1-kb promoter fragment K19 (GenBank, AF237661) amplified by genomic PCR, a 1.0-kb SV40 polyA cassette and a synthetic chimeric intron excised from pCI (Promega, Madison, WI, USA) were cloned into pBluescript vector (Stratagene, La Jolla, CA, USA) to construct pK19. Full-length cDNAs for COX-2 and mPGES-1 were amplified by RT–PCR. After sequence confirmation, the cDNA fragments were subcloned into pK19 to construct pK19-COX-2 and pK19-mPGES, respectively. Two expression vectors were co-microinjected into fertilized eggs of the F_1 (C3H and C57BL/6) hybrid females crossed with C57BL/6 male. Two of the six constructed transgenic lines, *K19-C2mE-2* and *K19-C2mE-8*, showed high expression levels of COX-2 and mPGES-1, and N2-backcrossed mice with C57BL/6 of these lines were used for further analysis. Wild-type littermates were used as controls.

Northern blotting

Total RNA (15 μg) extracted from glandular stomach was electrophoresed in 1% agarose, transferred to Hybond-N$^+$ nylon filters (Amersham, Little Chalfont, UK), hybridized with the [^{32}P]-labeled COX-2 or mPGES-1 cDNA probe, and autoradiographed.

Histopathology and immunohistochemistry

Tissues were fixed in 4% paraformaldehyde, embedded and sectioned at 4-μm thickness. These sections were stained with H&E and processed for further staining. Mucins were visualized by staining with Alcian blue (pH 2.5) or FITC-labeled *Helix pometia*-lectin (Sigma, St. Louis, MO, USA). For immunohistochemistry, rabbit polyclonal antibodies for COX-2 and mPGES-1 (Cayman Chemical, Ann Arbor, MI, USA), rat monoclonal antibodies for F4/80 (Serotec, Oxford, UK), CD11c, CD3ε and class II antigen (BD: Becton Dickinson, Franklin Lakes, NJ, USA), mouse monoclonal antibody for CXCL14 (BD) and goat polyclonal antibody for TNF-α (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used as the primary antibodies. Staining signals were visualized using the Vectorstain Elite Kit (Vector, Burlingame, CA, USA). The MOM. Kit (Vector Laboratories, Burlingame, CA, USA) was used to minimize the background staining signals. For immunofluorescence staining, Alexa Fluor anti-goat IgG (Molecular Probes, Eugene, OR, USA) or FITC-conjugated anti-rat IgG (Jackson Immunoresearch, West Grove, PA, USA) was used for the secondary antibody.

PGE_2 analysis

The PGE_2 levels were measured at SRL (SRL, Inc., Tokyo, Japan). The gastric mucosa was homogenized in a lysis buffer, extracted with ethanol and the PGE_2 levels were measured by radioimmunoassay (RIA) using [^{125}I]-labeled PGE_2 (Perkin Elmer, Boston, MA, USA). Culture supernatants were used directly after appropriate dilutions.

BrdU-labeling index

Mice were injected i.v. with 200 μl of BrdU solution (Roche Diagnostics, IN, USA) 1 h before euthanasia. Tissue samples were fixed in 70% ethanol, embedded and sectioned at 5-μm thickness. These sections were stained with anti-BrdU antibody (Roche). The labeling index was calculated by dividing the number of BrdU-positive cells with the total number of nucleated cells.

Treatment of mice with chemicals

For COX-2 inhibition, mice were injected subcutaneously with 10 mg/kg/day of NS-398 (Sigma) in 5% Arabia gum for 4 weeks starting at 16 weeks of age. For antibiotics, mice were treated for 3 weeks from 17 weeks of age with streptomycin (5 mg/ml in drinking water) and cefoperazone (100 mg/kg/day, s.c. injection) (Sigma) as previously described (Zavros *et al*, 2002b). For inhibition of proinflammatory cytokines, such as TNF-α, IL-1 and IL-6, mice of 20 weeks of age were injected i.p. with 80 mg/kg of pentoxifylline (Sigma) every 12 h for 5 days (Voisin *et al*, 1998).

Bacterial counts

Glandular stomach was homogenized in sterilized saline. Serially diluted homogenates were spread on trypticase soy agar plates with 5% sheep blood (BBL: Becton Dickinson Labware, Sparks, MD, USA) and incubated under aerobic, microaerophilic and anaerobic conditions at 37°C for 1–3 days. For microaerophilic and anaerobic conditions, Campy Pouch and GasPak Pouch (BBL) were used, respectively. The colony-forming units (CFU)/glandular stomach were calculated from triplicated experimental results.

Cell culture experiments

Gastric epithelial cells were prepared from mice at 3 weeks of age and cultured as described (Fujikawa *et al*, 2003). Mouse monocyte cell line RAW264 was used for positive control (RIKEN BioResource Center, Tsukuba, Japan). Cells were treated with LPS from *Salmonella typhimurium* (Sigma) or PGN from *Staphylococcus aureus* (Fluka) for 20 h, and TNF-α concentration in culture supernatants was measured using an ELISA kit (Biosource, Camarillo, CA, USA). For blocking the TLR4 signaling, monoclonal antibody to mouse TLR4/MD2 (Clone MTS510; HyCult biotechnology, Uden, Netherlands) was used at 10 μg/ml. *H. felis* (ATCC 49179) was cultured as described (Fox *et al*, 1996). For *H. felis* infection, 0.5% horse serum was used to reduce nonspecific basal COX-2 expression. Bacteria were inoculated to epithelial cells at moi 100, and co-cultured for 20 h. For RT–PCR analysis of COX-2 expression, primary epithelial cells were cultured in 0.5% horse serum-containing medium.

LMD system

Tissues were frozen immediately after euthanasia and embedded in O.C.T. compound (Tissue-Tek, Torrance, CA, USA). Gastric mucosal regions were excised from frozen sections using LMD (Leica Microsystems, Wetzler, Germany). Total RNA was extracted and processed for RT–PCR analysis. For *H. felis* infection experiments, female C57BL/6 mice were infected orally with 5.0×10^8 cfu per mice. Tissues were sampled 6 h after infection and surface epithelial layers of glandular stomach were excised with LMD.

RT–PCR analysis

Total RNA was extracted from the glandular stomach and primary culture epithelial cells, using ISOGEN (Nippon Gene, Tokyo, Japan). Extracted RNA was reverse-transcribed and PCR-amplified. Band intensities of the RT–PCR products were quantified in a densitometer, using Image J (NIH, Bethesda, MD, USA). Specific GAPDH primers were used for the internal control to normalize the sample amounts. RT–PCR was carried out using the following primers: COX-2 (F-5′-CAAACTCAAGTTTGACCCAG-3′, R-5′-GCCGGGATCCTTTTACAGCTCAGTTGAACG-3′), mPGES-1 (F-5′-CCGAATTCTTGAAGTCCAGGCCGGCTAG-3′, R-5′-TAATGTCGACACCAAGTCCGCAAGTTC3′), TLR2 (F-5′-TAAGCTGTGTCTCCACAAGC-3′, R-5′-CTCCAGGTAGGTCTTGGTGT-3′), TLR4 (F-5′-GTGTGTCAGTGATCAGTGTG-3′, R-5′-GTCTTCTCCAGAAGATGTGC-3′), TNF-α (F-5′-GTGACAAGCCTGTAGCCCA-3′, R-5′-AAAGTAGACCTGCCCGGAC-3′), IL-1β (F-5′-ATTAGACAACTGCACTACAGGCTC-3′, R-5′-GGATTCCATGGTGAAGTCAATTAT-3′), IL-6 (F-5′-CATGTTCTCTGGGAAATCGTGG-3′, R-5′-TGCCGAGTAGATCTCAAAGTG-3′), MIP-2 (F-5′-CTGTTGTGGCCAGTGAACTGCG-3′, R-5′-GGCTCCTCCTTTCCAGGTCAGT-3′), CXCL14 (F-5′-GCGTTGGACGGGTCCAAGTGT-3′, R-5′-TTCGTAGACCCTGCGCTTCT-3′), CCL2 (F-5′-TGTCATGCTTCTGGGCCTGCT-3′, R-5′-TTCACTGTCACACTGGTCACT-3′), CCL3 (F-5′-GGTCTCCACACTGCCCTT-3′, R-5′-TCAGGCATTCAGTTCCAGGTC-3′), CCL4 (F-5′-GAAGCTCTGCGTGTCTGCCCT-3′, R-5′-ACTCCAAGTCACTCATGTACT-3′), CCL5 (F-5′-ATGAAGATCTCTGCAGCTGCC-3′, R-5′-CTAGCTCATCTCCAAATAGTT-3′), GM-CSF (F-5′-GCATTGTGGTCTACAGCCTCT-3′, R-5′-GCTGTCTATGAAATCCGCATA-3′), HGF (F-5′-GGCTTGGCATCCACGATGTTC3′, R-5′-CCAGGACGATTTGGGATGGCA-3′) and VEGF (F-5′-GCCAAGTGGTCCCAGGCTGC-3′, R-5′-CTGTGCTGTAGGAAGCTCAT-3′).

Chemotaxis assay

Cell migration was assayed in Transwell culture chambers (5-μm-pore membranes, Coster, Cambridge, MA, USA). RAW264 cells were cultured in the presence or absence of 1 μM PGE_2 for 20 h. Then, cells (5×10^4) were added to the upper chamber, whereas recombinant mouse BRAK/CXCL14 (R&D systems, Inc., Minneapolis, MN, USA) was added to the lower chamber at 0, 100 and 400 ng/ml. After incubation for 1 h, cells attached on the lower surface of the membrane were counted in at least five different fields (original magnification, ×200).

Statistical analysis

Statistical analyses were carried out by unpaired Student's *T*-test, and *P*-value <0.05 was considered significant.

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