

Review

## Chemoprevention of *Helicobacter pylori*-associated Gastric Carcinogenesis in a Mouse Model; Is It Possible?

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Although debates still exist whether *Helicobacter pylori* infection is really class I carcinogen or not, *H. pylori* has been known to provoke precancerous lesions like gastric adenoma and chronic atrophic gastritis with intestinal metaplasia as well as gastric cancer. Chronic persistent, uncontrolled gastric inflammations are possible basis for ensuing gastric carcinogenesis and *H. pylori* infection increased COX-2 expressions, which might be the one of the mechanisms leading to gastric cancer. To know the implication of long-term treatment of antiinflammatory drugs, rebamipide or nimesulide, on *H. pylori*-associated gastric carcinogenesis, we infected C57BL/6 mice with *H. pylori*, especially after MNU administration to promote carcinogenesis and the effects of the long-term administration of rebamipide or nimesulide were evaluated. C57BL/6 mice were sacrificed 50 weeks after *H. pylori* infection. Colonization rates of *H. pylori*, degree of gastric inflammation and other pathological changes including atrophic gastritis and metaplasia, serum levels and mRNA transcripts of various mouse cytokines and chemokines, and NF- $\kappa$ B binding activities, and finally the presence of gastric adenocarcinoma were compared between *H. pylori* infected group (HP), and *H. pylori* infected group administered with long-term rebamipide containing pellet diets (HPR) or nimesulide mixed pellets (HPN). Gastric mucosal expressions of ICAM-1, HCAM, MMP, and transcriptional regulations of NF- $\kappa$ B binding were all significantly decreased in HPR group than in HP group. Multi-probe RNase protection assay showed the significantly decreased mRNA levels of apoptosis related genes and various cytokines genes like IFN- $\gamma$ , RANTES, TNF- $\alpha$ , TNFR p75, IL-1 $\beta$  in HPR group. In the

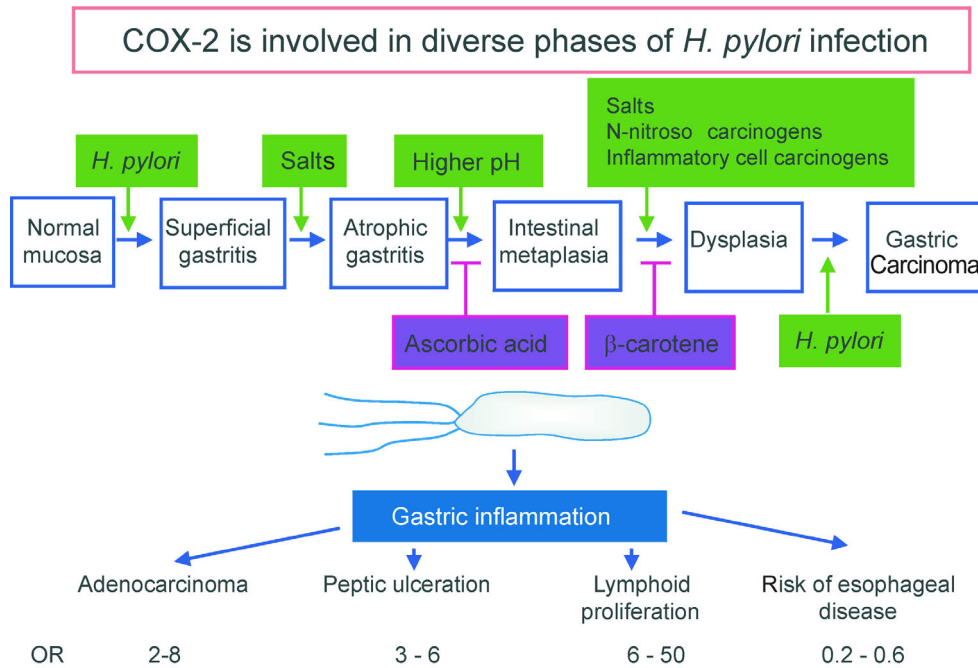
experiment designed to provoke gastric cancer through MNU treatment with *H. pylori* infection, the incidence of gastric carcinoma was not changed between HP and HPR group, but significantly decreased in HPN group, suggesting the chemoprevention of *H. pylori*-associated gastric carcinogenesis by COX-2 inhibition. Long-term administration of antiinflammatory drugs should be considered in the treatment of *H. pylori* since they showed the molecular and biologic advantages with possible chemopreventive effect against *H. pylori*-associated gastric carcinogenesis. If the final concrete proof showing the causal relationship between *H. pylori* infection and gastric carcinogenesis could be obtained, that will shed new light on chemoprevention of gastric cancer, that is, that gastric cancer could be prevented through either the eradication of *H. pylori* or lessening the inflammation provoked by *H. pylori* infection in high risk group.

**Keywords:** Antiinflammation, Apoptosis, Chemoprevention, C57BL/6 mouse, Cytokines, *Helicobacter pylori*, Nimesulide, Rebamipide

### The Causal Relationship and Mechanism between *H. pylori* Infection and Gastric Pathologies

The gastric inflammatory response induced by *H. pylori* consists of neutrophils, lymphocytes, plasma cells, and macrophages, along with varying degrees of epithelial cell degeneration and injury (Israel and Peek, 2001). As mechanisms by which *H. pylori* provoke gastric inflammations, the following two possibilities had been largely proposed. That is, one is that *H. pylori* secrete substances that stimulate mucosal inflammation from afar (Mai *et al.*, 1992) and another mechanism is through direct

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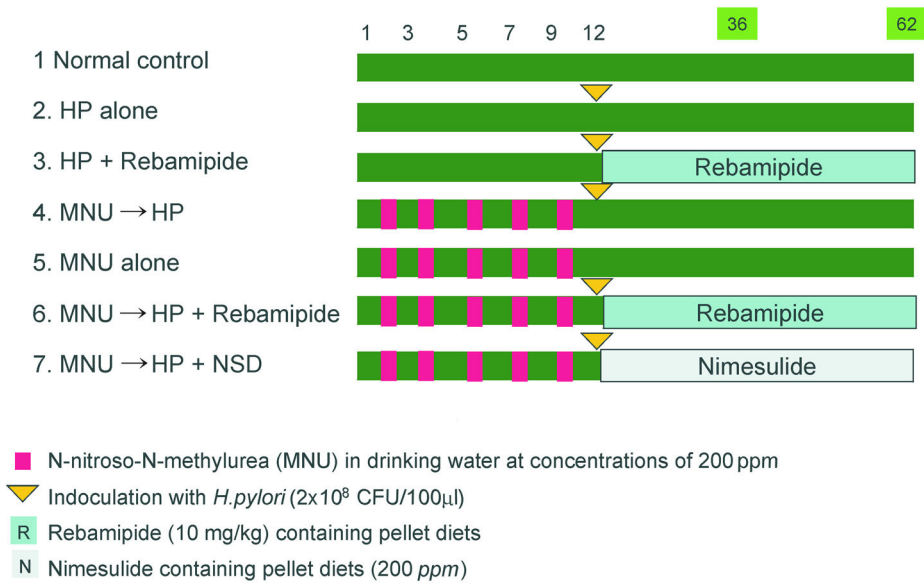
**Fig. 1.** Involvement of COX-2 in the various stages of *H. pylori* infection, providing insights about chemoprevention through the reduction of COX-2. *H. pylori* infection provoked superficial gastritis, atrophic gastritis, gastric dysplasia, and even gastric carcinoma, which led to the WHO's definition *H. pylori* as a class I carcinogen (1994 IARC, Lyon, France). The odd ratio of risk of gastric cancer by the *H. pylori* infection is 2-8. Interestingly, the *H. pylori* infection induced COX-2 expressions. Since COX-2 is largely regarded as responsible for tumorigenesis, the modulation of COX-2 levels or other inflammatory mediators might be intervening in the chemoprevention of the *H. pylori*-associated gastric carcinogenesis.

contact with gastric epithelial cells (through type IV secretory system) and stimulation of various cytokine releases (Yamaoka *et al.*, 1996; Go, 1997), based on the fact that gastric epithelia from *H. pylori*-infected persons demonstrate enhanced levels of interleukin-1 $\beta$  (IL-1 $\beta$ ), IL-2, IL-6, IL-8, interferon- $\gamma$  (IFN- $\gamma$ ), and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) (Yoshida, 1993; Moss *et al.*, 1994).

Another consequence of persistent inflammation may be alterations in cellular turnover. Gastric epithelial cell proliferation rates within colonized mucosa are significantly increased compared to those in uninfected controls (Bechi *et al.*, 1996). In chronic *H. pylori* infection, there is a notable lack of epithelial cell necrosis, suggesting that other forms of cellular demise such as apoptosis may be induced (Peek *et al.*, 1997). Although substantial variation exists among apoptosis scores in *H. pylori* infection, several studies have reported increased levels of gastric epithelial cell apoptosis among *H. pylori*-infected persons (Moss *et al.*, 1996). *H. pylori*-induced gastritis may also lead to other forms of epithelial cell injury and damage. Activated neutrophils generate reactive oxygen or nitrogen species that can induce oxidative DNA damage via formation of DNA adducts (Rautelin *et al.*, 1994; Zhang, 1996). Upon contact with *H. pylori* *in vitro*, an oxidative burst occurs within polymorphonuclear cells. The specific types of cellular damage resulting from generation of reactive oxygen species include lipid peroxidation, protein oxidation, and

oxidation of DNA (Baik *et al.*, 1996). All these mechanisms were principally involved in mucosal cell damages by *H. pylori* and some of these actions might be involved in *H. pylori*-associated gastric carcinogenesis. Therefore, some modifying trials will be needed to reduce the harmful injuries by *H. pylori* infection. The eradication of *H. pylori* infection itself or administration of blockers like NSAIDs or other anti-inflammatory drugs will be the next candidate.

Nimesulide is one of the preferred inhibitor of cyclooxygenase-2 (COX-2) prescribed for anti-inflammation and pain relief (Davis *et al.*, 1994). Several studies suggested these NSAIDs shed the possibility of chemoprevention in colon carcinogenesis and preventing the development of colon adenoma or polyps (Reddy, 1996; Jacoby *et al.*, 2000). Since COX-2 is highly expressed and induced in *H. pylori* infection and the attenuation of its expression was noted after the eradication of *H. pylori* (Sung *et al.*, 2000), COX-2 inhibitor will be also one of the candidate for potential chemopreventive agent in *H. pylori*-associated gastric carcinogenesis (Fig. 1). Rebamipide has been reported to improve *H. pylori*-associated gastritis through either removing active oxygen species, scavenger of active oxygen species, or attenuating the productions of cytokines/chemokines (Yoshida, 1996; Arakawa *et al.*, 1998). Until now although there have been several reports to document the effects and mechanisms of rebamipide in *H. pylori*-associated gastritis



**Fig. 2.** Protocol for the study. Out of a total of 220 C57BL/6 mice, 180 mice were divided into 6 groups of 30 mice in each group (Groups 3 and 6 allocated 40 mice per group, due to the relatively low colonization rate of *H. pylori* than the other groups). They were given MNU that was mixed in their drinking water at concentrations of 200 ppm (Groups 4-7) for a total of five cycles of one-week regimens with a one-week pause. After completion of the MNU administration, they were given autoclaved distilled water for two weeks. Groups 4, 6, and 7 were further inoculated with *H. pylori* three times every other day. *H. pylori* was given to mice without prior MNU treatment (Groups 2 and 3). The remaining 20 mice were given only distilled water, without *H. pylori* or MNU as non-carcinogen controls (Group 1).

and peptic ulcer disease (Yamasaki *et al.*, 1987; Han, 1995), all these were done on designated periods of administration. Therefore, none was reported about the *ex vivo* effects of long-term administration of rebamipide or COX-2 inhibitors on *H. pylori*-associated gastritis. Recently we did do animal experiment to document the chemopreventive activities of anti-inflammatory drugs on *H. pylori*-associated gastric carcinogenesis, which will appear in the following section.

### Experiments Performed to Document Whether the Long-term Administration of Anti-inflammatory Drugs Can Modify the Process of MNU and *H. pylori*-associated Gastric Carcinogenesis

Based on the assumption that chronic, persistent inflammation might predispose to carcinogenesis (Ernst, 1999), oxidative DNA damages and COX-2 expressions provoked by *H. pylori* infection could be reduced through the eradication of *H. pylori*, especially more effectively by the drug regimen including decreasing oxidative stress (Hahm *et al.*, 1997), and gastric adenocarcinoma could be generated after long-term *H. pylori* infection in Mongolian gerbils or after co-administration of *H. pylori* and N-methyl-N-nitrosourea (MNU) (Sugiyama *et al.*, 1998), we compared the pathological changes, changes of cellular proteins known to be involved in either inflammation or carcinogenesis and mRNA transcripts of cytokine and chemokine, and apoptosis

related genes among normal controls, *H. pylori*-infected group, and long-term administration of anti-inflammatory drugs, nimesulide or rebamipide, after *H. pylori* infection. Since we could not observe the development of gastric cancer in mice model with *H. pylori* infection alone (Han *et al.*, 2002), we added another set of experiment including MNU pretreatment with or without additional *H. pylori* infection for the current experiment. A total of two hundred twenty specific pathogen free, six-week-old female C57BL/6 mice were housed in steel cages on hard wood chip bedding in an air-conditioned biohazard room with a 12 hr light-12 hr dark cycle. N-methyl-N-nitrosourea (MNU) was prepared freshly twice a week by dissolving in distilled water at concentrations of 200 p.p.m. The solutions were administered as drinking water in light-shielded bottles *ad libitum*. The mouse-adapted *H. pylori* (SS1, Sydney strain, kind gift of professor Adrian Lee), originally isolated from a peptic ulcer patient, were inoculated on Brucella agar plates containing 10% heat-inactivated fetal bovine serum and Skirrow medium. They were kept at 37°C under micro-aerobic conditions using GasPak jar and CampyPak. After 24 hr of fasting, 0.1 ml suspension of *H. pylori* containing  $1 \times 10^8$  colony-forming units (CFU)/ml were delivered to mice intragastrically using an oral catheter. Rebamipide was administered mixed in chow pellet diets. According to preliminary data of Otsuka Pharmaceutical Research Center, treatment of rebamipide 20 mg/kg/day maintained 0.02% blood concentration of rebamipide. Nimesulides, a preferred COX-2 inhibitor (Davis

*et al.*, 1994), were also given CRF-1 diet containing 0.02% nimesulide. The drug was mostly biotransformed into 4-hydroxynimesulide, a metabolite with appears to contribute to the anti-inflammatory activity of the compound. The mean plasma concentrations of nimesulide became peak after 2-4 hours after administration. Since there was tendency mice refuse to take these pellet diets containing test drugs, the amounts consumed were monitored daily. Out of total two hundreds twenty C57BL/6 mice, 180 mice were divided into 6 groups of 30 mice in each group (Group 2 and Group 5 allocated 40 mice per group due to low colonization rate of *H. pylori*) and were given MNU mixed in their drinking water at concentrations of 200 p.p.m. (Group 4-7) for total five cycles of one-week regimen with one-week pause. After completion of MNU administration, they were given autoclaved distilled water for two weeks, and group 4, 6, and 7 were further inoculated with *H. pylori* three times every other day. *H. pylori* was given to 20 mice without prior MNU treatment (Group 2 and 3), and remaining 20 mice were given only distilled water without neither *H. pylori* nor MNU as non-carcinogen controls (Group 1). At the time points of 16 to 24 weeks after *H. pylori* administration, 10 mice from each group were sacrificed only to look at the presence of *H. pylori* by direct cultures and CLO test. The observation period was extended up to 50 weeks after *H. pylori* infection. These experimental protocols were summarized in Fig. 2.

All animals were sacrificed under deep ether anesthesia at 50<sup>th</sup> week after the *H. pylori* infection. The stomachs, opened along the greater curvature, were fixed in 10% neutralized formalin in phosphate buffered saline, processed by standard methods, and embedded in paraffin. Tissues were sectioned at

4  $\mu$ m for hematoxylin and eosin staining. Histological typing of adenocarcinoma was based on the predominant pattern of tumor and the lesions of the glandular stomach were classified by the following criteria. Tubular adenocarcinomas were defined as tumor cells with cellular atypia, nuclear anisonucleosis, hyperchromatism, thickened and irregular nuclear membrane, and stromal invasion or architectural destruction. Signet ring cell carcinomas were defined as isolated tumor cells containing large amounts of mucins. Adenoma was defined as proliferative immature glandular epithelium with varying degrees of dysplasia and foveolar hyperplasia was defined as an increased height of the pit with hyperchromatic nuclei and mitotic activity. The presence of intestinal metaplasia and gastric atrophy was also measured. In order to make the expression of each group represent that of each group, gastric mucosal scratches from five mice/group were mixed together and were homogenized in iced cold RIPA buffer. After fractionating in 4-12.5% SDS/PAGE, the proteins were transferred to nitrocellulose filters. The kinds of antibodies used for the current experiment were all purchased from Santa Cruz Biotechnology except pS2/TFF1 antibody, gift from Dr. G. Elia (Clone GE1, 31AA, London, UK). They were intercellular adhesion molecule-1 (ICAM-1), HCAM (CD44); a glycoprotein "homing receptor", transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1); an anti-inflammatory cytokine, heat shock protein 70 (Hsp 70); molecular chaperon, MDM-2; a regulatory gene of p53, proliferating cell nuclear antigen (PCNA), Bcl-2, Bax, cyclooxygenase-2 (COX-2), *h-met*; a receptor molecule for hepatocyte growth factor, ornithine decarboxylase (ODC); a rate-limiting enzyme for gastric polyamine synthesis. The RPA is a highly sensitive and

**Table 1.** Incidence of glandular gastric tumors of mice according to group

Group/Treatment	Total No. of mice	Effective No. of mice	Dead No. of mice (%)	No. of tumor bearing mice (% incidence)
2. HP alone	20	19	1 ( 5)	0
3. HP + Rebamipide	20	20	0	0
4. MNU + HP	20	17	3 (15)	11 (64.7)
5. MNU alone	20	19	1 ( 5)	2 (10.5)*
6. MNU + HP + Rebamipide	20	18	2 (10)	11 (61.1)
7. MNU + HP + NSD	20	18	2 (10)	7 (38.9)**

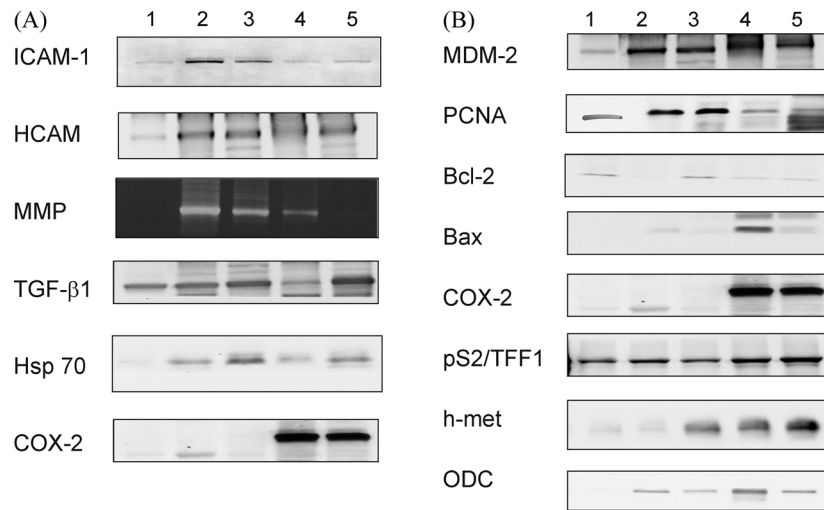
\*Group 4 vs Group 5 (p<0.01)

\*\*Group 4 vs Group 7 (p<0.01)

**Table 2.** Incidence of glandular gastric tumors by histologic findings

Group/Treatment	Effective No of mice	No. of Tumor Bearing mice	Adenoma (%)	Adenocarcinoma (%)
4. MNU $\rightarrow$ HP	17	11 (64.7)	4 (23.5)	7 (41.2)
5. MNU alone	19	2 (10.5)	1 ( 5.3)	1 ( 5.3)
6. MNU $\rightarrow$ HP + Rebamipide	18	11 (61.1)	5 (27.8)	6 (33.3)
7. MNU $\rightarrow$ HP + NSD	18	7 (38.9)	6 (33.3)	1 ( 5.6)*

\*Group 4 vs Group 7 (p<0.01)

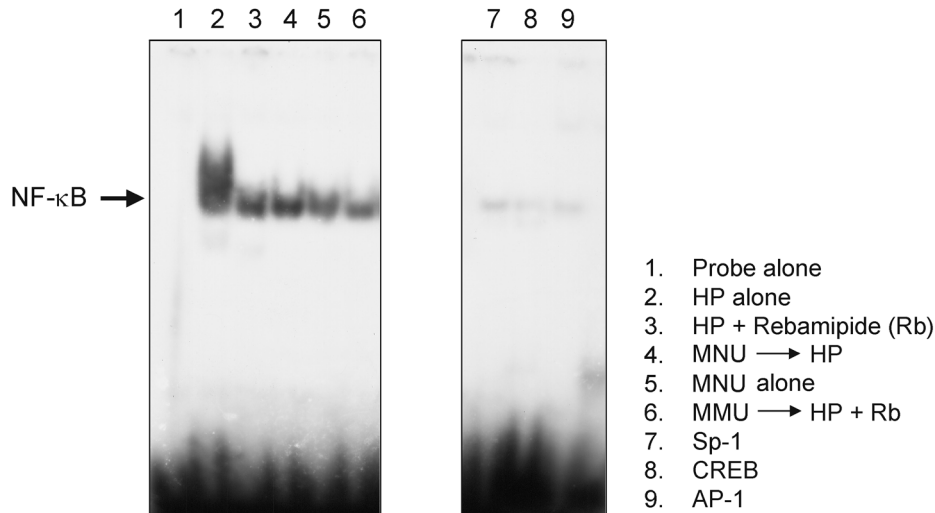


**Fig. 3.** Changes of protein profiles involved in (A) inflammation and (B) carcinogenesis. (A) Compared to the normal controls, the *H. pylori*-infected stomach showed increased expressions of ICAM-1, HCAM, MMP, and Hsp70. Lane 3 (denoted *H. pylori*-infected mice that were administered with long-term rebamipide) showed the marked attenuation of ICAM-1 and MMP, and increased expressions of TGF- $\beta$ 1 and Hsp 70, more than lane 2 did, which signifies that the long-term administration of rebamipide decreased proteins, which are involved in the propagation or perpetuation of inflammation. (B) The *H. pylori* infection increased the expressions of MDM-2, PCNA, and ODC. Compared to lane 4 (MNU + *H. pylori*), lane 5 (MNU + *H. pylori* with long-term administration of rebamipide) showed decreased expressions of MDM-2, PCNA, Bax, and ODC. On the other hand, the expressions of pS2/TFF1 and h-met increased significantly more in lane 5 than in lane 4. Lane 1; Normal control, Lane 2; *H. pylori* alone, Lane 3; *H. pylori* + rebamipide, Lane 4; MNU + *H. pylori*, Lane 5; MNU + *H. pylori* + rebamipide.

specific method for the detection and quantitation of mRNA species. The probe used for RNase protection assay was synthesized using protocol provided with RiboQuant<sup>®</sup> *in vitro* transcription kit, using 100 mCi of 800 Ci/ mmol [ $\alpha$ -<sup>32</sup>P] UTP and the probes purchased from PharMingen, named multi-probe RNase protection assay system. They used mCK-2 containing interleukin-12p35 (IL-12p35), IL-12p40, IL-10, IL-1 $\beta$ , IL-1 $\alpha$ , monokine induced by IFN- $\gamma$  (MIG), IL-6, and interferon-gamma (IFN- $\gamma$ ), mCK-3 containing tumor necrosis factor-beta (TNF- $\beta$ ), lymphotoxin-beta (LT- $\beta$ ), TNF- $\alpha$ , IL-6, IFN- $\beta$ , IFN- $\alpha$ , transforming growth factor-beta1 (TGF- $\beta$ 1), TGF- $\beta$ 2, mCK-5 containing lymphotactin (Ltn), Regulated on activation, normal T-cell expressed and secreted (RANTES), Eotaxin, macrophage inflammatory protein-one beta (MIP-1 $\alpha$ ), MIP-1 $\beta$ , MIP-2, IFN- $\gamma$  inducible protein 10 (IP-10), monocyte chemoattractant protein 1 (MCP-1), T cell activation protein-3 (TCA-3), mAPO-3 containing caspase-8, FasL, Fas, Fas-associated death domain (FADD), Fas-associated phosphatase (FAP), Fas-associated factor (FAF), TNF-related apoptosis inducing ligand (TRAIL), TNFRp55, TNFR1-associated death domain protein (TRADD), receptor-interacting protein (RIP).

**Effect of the long-term administration of nimesulide or rebamipide on tumor incidence of mice** The survival rate of each group was more than 85% and all animals in Group 3 survived until the 50th week (Table 1). The incidence of gastric tumors at the 50th week was 64.7%, 10.5%, 61.1%, and 38.9% in Group 4, Group 5, Group 6, and Group 7, respectively,

suggesting the significantly higher incidence of gastric tumors in mice treated with the combination of MNU and *H. pylori* infection and significantly lower incidence of gastric tumors in group co-treated with nimesulide. These results signified that *H. pylori* infection surely promoted MNU-induced gastric carcinogenesis and these promoting effect was abolished by COX-2 inhibitor. *H. pylori* alone (Group 2) or *H. pylori* administered with long-term rebamipide (Group 3) didn't develop any gastric tumors in mice model at 50 weeks. On the pathological evaluation of gastric tumors developed in Group 4-7, gastric adenocarcinoma were noted in 41.2% of Group 4, 5.3% of Group 5, 33.3% of Group 6, and 5.6% of Group 7 (Table 2). No significant findings except chronic atrophic gastritis and foveolar hyperplasia were observed in Group 2 (50th week). This finding means that *H. pylori* infection might play a definite role as promoter in gastric carcinogenesis rather than direct carcinogen. Gastric adenomas showing proliferative immature glandular epithelium with varying degrees of dysplasia were frequently found in the groups treated with MNU and *H. pylori* infection, whereas gastric adenoma was observed in only one mouse of MNU alone. About two-thirds of adenocarcinoma was tubular adenocarcinoma and the remaining one-third was signet ring cell carcinoma. Most of the tumors (18/23) were found in the antrum. All these findings including gastric histopathologies were summarized in Fig. 7. In contrast to no significant pathological findings in uninfected mice, chronic atrophic gastritis and foveolar hyperplasia were seen in the mucosa of all *H. pylori* infected mice at the 50th week. In *H. pylori*-infected mice, marked infiltrations of



**Fig. 4.** Effects of long-term rebamipide on NF-κB binding EMSA of the NF-κB, Sp-1, CREB, and AP-1 showed increased DNA bindings after the *H. pylori* infection, more than the non-infected mice did (lane 1). The nuclear proteins of each lane were extracted from the three randomly-chosen-mice stomachs of each group, suggesting the EMSA band as the mean activity of each group. Treatment of rebamipide (lanes 3 & 6) significantly decreased NFκB-DNA binding, more than did lanes 1 or 4/5.

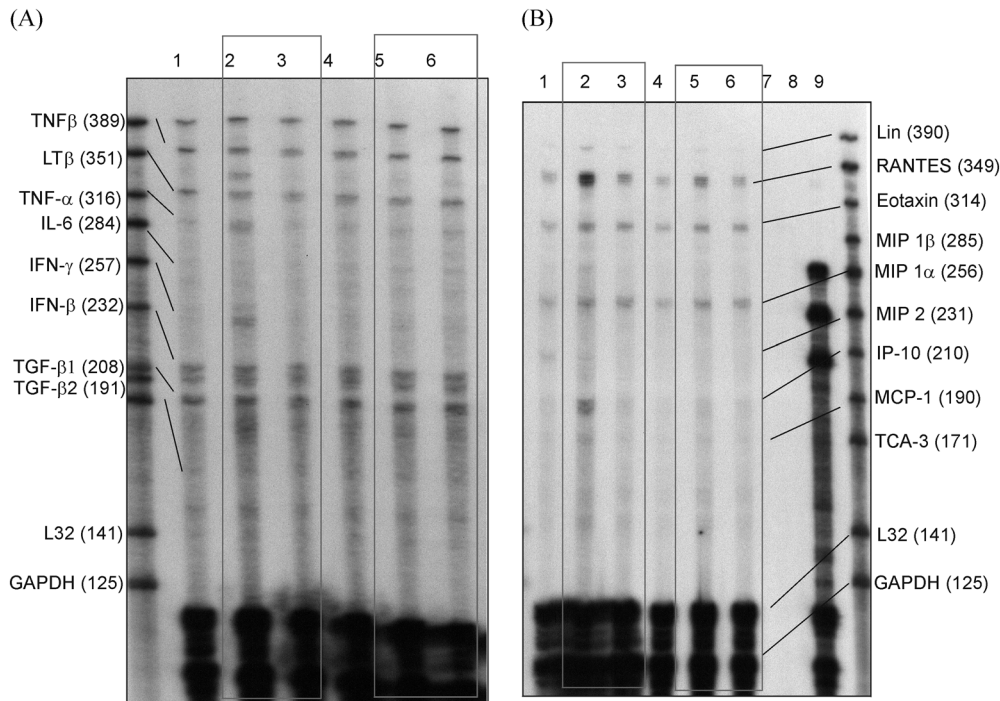
lymphocyte, plasma cells, and neutrophils and eosinophils with lesser frequency were present in the lamina propria and submucosa and formations of lymphoid follicles. Interestingly, Group 6, HPR, and Group 7, HPN, showed marked low incidence of atrophic gastritis compared to Group 4, MNU + *H. pylori*, suggesting long-term rebamipide or nimesulide administration reduced the development of chronic atrophic gastritis and intestinal metaplasia (data not shown).

**Effects of the long-term administration of rebamipide on the expressions of various gastric proteins** We divided the various proteins tested into two groups based on their implication of action, inflammation related proteins (Fig. 3A) and cell growth related proteins (Fig. 3B). After *H. pylori* infection, ICAM-1, HCAM (CD44), MMP, COX-2, and Hsp 70 were induced or increased definitely (lane 2 in Fig. 3A and 3B). On the other hand, long-term administration of rebamipide mixed in pellet diets caused the attenuation of the expressions of ICAM-1, HCAM, MMP and increased the expressions of Hsp 70 (lane 3 in Fig. 3A and 3B). These results suggested the excellent actions of rebamipide in either decreasing the proteins involved in the propagation and aggravation of gastric inflammation or increasing cytoprotective protein. TGF-β1, well-known anti-inflammatory cytokine, was modestly increasingly expressed in Group 6, MNU + *H. pylori* + rebamipide. COX-2 expression was not prominent irrespective of rebamipide or *H. pylori*, but significantly increased after MNU + *H. pylori* group. In group 4, MNU + *H. pylori*, ornithine decarboxylase (ODC), *Bax*, pS2/TFF1 (trefoil peptide protein), *h-met*, and COX-2 were significantly increasingly induced, suggesting the increased cellular proliferations, but the levels of expression of ODC, COX-2, PCNA were significantly

decreased after the further chronic administration of rebamipide. pS2/TFF1, a tumor suppressor protein specially engaged in gastric carcinogenesis, was slightly increasingly expressed after long-term administration of rebamipide.

**Effects of the long-term administration of rebamipide on NF-κB binding** Fig. 4 showed the NF-κB complex of nuclear proteins extracted from each group detected by electrophoretic mobility shift assay. The nuclear proteins were extracted from the three randomly selected mice stomach of each group, suggesting the EMSA band as the mean activities of each group. *H. pylori* infection (lane 2) increased NF-κB-DNA binding significantly than non-infected group (lane 1). However, treatment of rebamipide decreased NF-κB binding significantly (16.6% of lane 2). In a point of NF-κB-DNA binding, administration of MNU only caused slight increment of NF-κB binding irrespective of *H. pylori* infection, but lane 6, group treated with rebamipide also attenuated the NF-κB binding than MNU and *H. pylori* group (lane 5), suggesting the possible actions of rebamipide on modulating the inflammatory response through transcriptional regulation of NF-κB.

**Effects of the long-term administration of rebamipide on mRNA of cytokines and apoptosis-related genes** We investigated the various mRNA transcripts of mouse cytokines and chemokines by multi-probe RNase protection assay. Fig. 5A showed the RPA of mCK-3 (RiboQuant®, multi-probe RPA) showing TNF-β, LTβ, TNF-α, IL-6, IFN-γ, IFN-β, TGF-β1, TGF-β2. Among them TNF-β, LTβ, TNF-α, IL-6 and IFN-γ were increased after chronic *H. pylori* infection. After long-term administration of rebamipide, all of these transcripts were significantly decreased (lane 2 vs lane 3). No



**Fig. 5.** Effects of long-term rebamipide on mRNA of cytokines genes (A) Multi-probe RPA of mouse cytokines [mCK-3 kit (RiboQuant®, PharMingen Cat # 45003p)] contained TNF- $\beta$ , LT $\beta$ , TNF- $\alpha$ , IL-6, IFN- $\gamma$ , IFN- $\beta$ , TGF- $\beta$ 1, and TGF- $\beta$ 2. Among them, TNF- $\beta$ , LT $\beta$ , TNF- $\alpha$ , IL-6, and IFN- $\gamma$  increased after chronic *H. pylori* infection, which was significantly decreased after the long-term administration of rebamipide. (B) Multi-probe RPA of mouse cytokines [mCK-5 kit (RiboQuant®, Cat # 45026p)] RPA of mCK-5 displayed chemokines, like Ltn, RANTES, Eotaxin, MIP-1 $\beta$ , MIP-1 $\alpha$ , MIP-2, IP-10, MCP-1, and TCA-3. Similar to (A), the *H. pylori* infection increased all of these chemokines mRNA, except MIP-2 and TCA-3. The long-term administration of rebamipide showed remarkable decreases in the mRNA of RANTES, Lin, and IP-10. Lane 1; Normal control, Lane 2; *H. pylori* alone, Lane 3; *H. pylori* + rebamipide, Lane 4, MNU alone, Lane 5; MNU + *H. pylori*, Lane 6; MNU + *H. pylori* + rebamipide, Lane 7; Yeast RNA, Lane 8; Control RNA, Lane 9; Size markers.

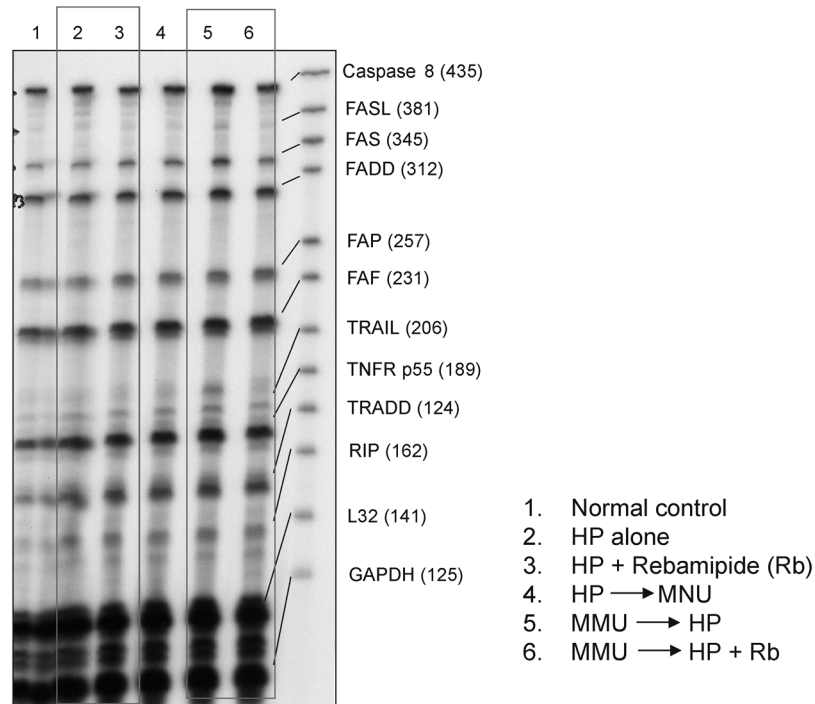
significant change was noted in MNU treated group irrespective of rebamipide administration. Fig. 5B showed the RPA of mCK-5 showing chemokines like Ltn, RANTES, Eotaxin, MIP-1 $\beta$ , MIP-1 $\alpha$ , MIP-2, IP-10, MCP-1, TCA-3. Similar to cytokines, *H. pylori* infection increased all of these chemokines except MIP-2 and TCA-3. Long-term administration of rebamipide showed remarkable decreases in transcript levels of RANTES, Lin, and IP-10 in MNU treated group, rebamipide decreased RANTES, eotaxin, and MIP-1 $\alpha$ . Fig. 6 showed the RPA of mAPO-3 containing caspase-8, FasL, Fas, FADD, FAP, FAF, TRAIL, TNFRp55, TRADD, RIP. *H. pylori* infection increased the genes involved in apoptosis. Slight decreases in transcript of caspase-8, FasL, Fas and TRAIL were observed in long-term administration of rebamipide pretreated with MNU.

### Chemoprevention of *Helicobacter pylori*-associated Gastric Carcinogenesis in Mice Model; Is It Possible?

Our above described study showed nimesulide, preferred COX-2 inhibitor, resulted in significant decrease in *H. pylori*-

associated gastric carcinogenesis and rebamipide, anti-ulcer and anti-inflammatory drug, showed significant attenuating effects in the severity of *H. pylori*-associated gastritis, based on the findings that rebamipide decreased cell adhesion molecule, ICAM-1 and HCAM, reduced MMP activities, decreased COX-2 expressions, reduced the DNA binding of inflammation-associated transcription factor, NF- $\kappa$ B, decreased cytokine activities like IL-1 $\beta$ , TNF- $\alpha$ , RANTES, IP-10, MIP-1 $\alpha$ , and IFN- $\gamma$ .

Long-term administration of nimesulide showed significant strong chemopreventive actions against *H. pylori*-associated gastric carcinogenesis and halted the progress from gastric adenoma to gastric cancer. These results might be ensued from the blockade of COX-2 in the progression of adenomatous growth. Whereas, the long-term administration of rebamipide did not show the definite evidence of chemoprevention in spite of excellent anti-inflammatory action, which could be explained by the fact that rebamipide maintained the balance between cell proliferation and apoptosis, since apoptosis must exceed cell proliferation in order to achieve chemoprevention (Moss, 1998). However, the development of precancerous lesion like chronic atrophic gastritis was surely retarded and decreased by the long-term



**Fig. 6.** Effects of long-term administration of rebamipide on mRNA of apoptosis-related genes. RPA of mAPO-3 (RiboQuant®, Cat #45355p) dealt with caspase-8, FasL, Fas, FADD, FAP, FAF, TRAIL, TNFRp55, TRADD, and RIP. The *H. pylori* infection increased the genes that were involved in apoptosis. Slight decreases in the transcript of caspase-8, FasL, Fas and TRAIL were observed in the long-term administration of rebamipide that was pretreated with MNU. Lane 1; Normal control, Lane 2; *H. pylori* alone, Lane 3; *H. pylori* + rebamipide, Lane 4, MNU alone, Lane 5; MNU + *H. pylori*, Lane 6; MNU + *H. pylori* + rebamipide.

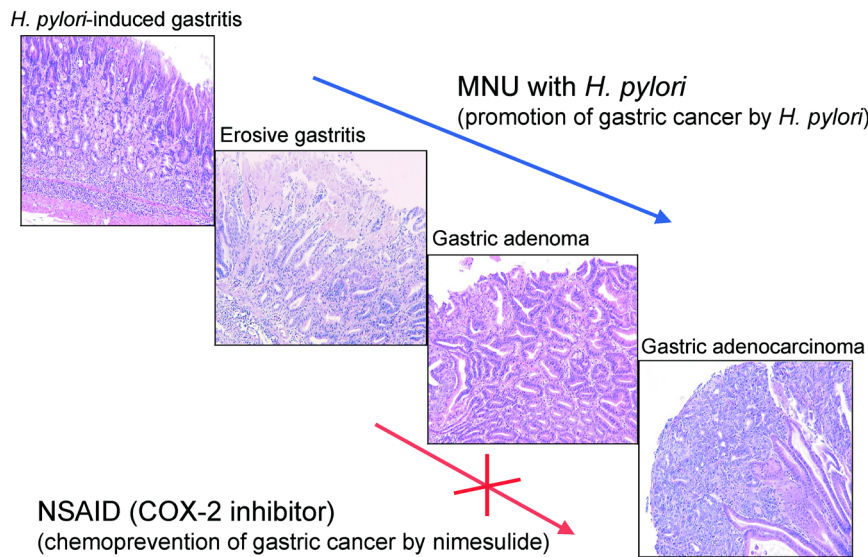
administration of rebamipide.

The study described in the above section might be the first study showing the chemoprevention of nimesulide or rebamipide in *H. pylori*-infected animal model (Fig. 7). Before this, there was rat model with chronic gastritis developed by the continuous administration of 5mM sodium taurocholate for six months. Rebamipide treatment for four weeks (6 and 60mg/kg/day) initiated after development of chronic gastritis dose-dependently attenuates the gastritis in terms of degree of inflammatory cell infiltration, erosions, and glandular atrophy (Kishimoto, 1992). Proposed mechanisms for anti-inflammatory and mucosal protective actions of rebamipide were decreased neutrophilic activity (Suzuki *et al.*, 1994), decreased production of inflammatory cytokines (Aihara *et al.*, 1998), and decreased generations of oxygen free radicals (Iinuma *et al.*, 1998). Chemoprevention by NSAIDs was only tried in familial adenomatous polyposis and colon cancer model. Chemoprevention trials in gastric carcinogenesis and even *H. pylori*-association were not reported at all before the current trial except *in vitro* evidence of retinoic acids in the cytotoxicity of gastric cancer cells. Recently Steinbach *et al.* (2000) reported the definite advantage of celecoxib, a specific COX-2 inhibitor, in preventing the development of colonic adenomatous polyposis in familial adenomatous polyposis patients. The exact underlying molecular mechanisms how nimesulide showed

the significant chemopreventive actions are under investigation. Instead, in the current study, data about the molecular actions of another antiinflammatory drug used in the current study, rebamipide, were revealed.

Since *H. pylori* generally adhered to gastric epithelial cells without invading the epithelium, it is presumed that an interaction between bacteria and host epithelia cells may initiate *H. pylori*-induced inflammation (Ernst *et al.*, 1997). Because of the strong inflammatory response to *H. pylori* infections, the role of inflammatory cytokines has been investigated and it was shown that mucosal biopsy specimens from patients with *H. pylori* infection contain significantly elevated levels of IL-1 $\beta$ , IL-6, TNF- $\alpha$ , and IL-8 compared to those in specimens from uninfected individuals (Crabtree, 1991; Rieder *et al.*, 1997; Yamaoka *et al.*, 2001). Most intracellular bacteria generally induce Th1 responses, while extracellular pathogens stimulate Th2 responses. Based on the fact that *H. pylori* are non-invasive and that infection is accompanied by an exuberant humoral response, one might predict that a Th2 response would be predominant within *H. pylori*-colonized gastric mucosa. Paradoxically, the majority of *H. pylori* antigen-specific T-cell clones isolated from infected gastric mucosa produce higher levels of IFN- $\gamma$  than IL-4 or IL-10, which is reflective of a Th1 type response (Bamford *et al.*, 1998). Therefore, certain strains of mice (C57BL/6) infected with *H. felis* that develop a Th1 type





**Fig. 7.** Pathological changes after MMU administrations and *H. pylori* infection. Serial changes of gastritis were observed after the *H. pylori* infection in mice. MNU and *H. pylori* infection promoted the development of gastric adenoma and adenocarcinoma. Without the *H. pylori* infection after the MNU administration, the pathologies remained as chronic atrophic or hypertrophic gastritis as far as the lifespan of mice. Nimesulide administration blocked the progression of gastric adenoma to gastric adenocarcinoma, and rebamipide administration decreased the development of precancerous lesions like chronic atrophic gastritis in mice. Photography ( $\times 100$  magnification)

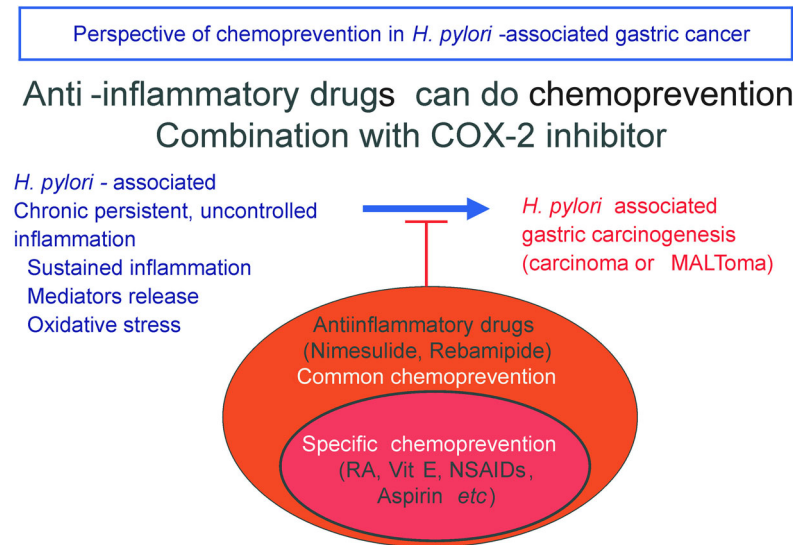
response exhibit extensive gastric inflammation, while other genetically distinct strains (BALB/c) that respond to infection with a Th2-like response develop only minimal gastritis (Sakagami *et al.*, 1996). Adoptive transfer of Th2 lymphocytes from infected mice into infected recipients reduces bacterial colonization density, while transfer of Th1 cells increases the severity of gastritis (Mohammadi *et al.*, 1997). In the current experiment, we could confirm that definite Th1 type response was generated by *H. pylori* infection and long-term administration of rebamipide, anti-inflammatory drug, attenuated Th1 type cytokine generations and increased Th2 type cytokine, which led to decreased gastric inflammation in final.

Cell-cell interactions play an important and probably central role in a large number of immunological processes in physiological and pathological conditions. These interactions are at least partially mediated by various cell adhesion molecules. Expression of cell adhesion molecules on gastric epithelial cells may participate in leukocyte homing and epithelial cell adhesions in *H. pylori*-associated mucosal inflammations (Mori *et al.*, 2000). In the context of *H. pylori* infection, the production of chemoattractive cytokines and cell adhesion molecules could provide a means of recruiting and retaining inflammatory cells within gastric epithelial cells, contributing to *H. pylori*-mediated tissue injury. Co-culture of epithelial cells with cytotoxin-associated gene pathogenicity island-positive (*cag* PAI+) *H. pylori* strains, but not with a *cag* PAI- strain resulted in up-regulation of steady-state mRNA levels and cell surface expressions of ICAM-1. *H. pylori* activated the ICAM-1 or IL-8 promoter via the NF- $\kappa$ B binding site (Keates *et al.*, 1997). In the current study, long-

term administration of rebamipide showed the advantage of decreasing ICAM-1 and even HCAM.

The finding that *H. pylori* is a potent activator of NF- $\kappa$ B has important implications, since other NF- $\kappa$ B responsive genes, including those encoding IL-8, TNF- $\alpha$ , IL-1, and IL-6, has been found to be elevated in the gastric mucosa of persons with *H. pylori* (Crabtree *et al.*, 1991; Sakagami *et al.*, 1996; Ernst *et al.*, 1997; Keates *et al.*, 1997; Mohammadi *et al.*, 1997; Rieder *et al.*, 1997; Aihara *et al.*, 1998; Bamford *et al.*, 1998; Iinuma *et al.*, 1998; Sharma, 1998; Mari *et al.*, 2000; Yamaoka *et al.*, 2001). NF- $\kappa$ B is well recognized as a redox-sensitive transcription factor and has been implicated in the cellular response to oxidative stress. Nearly all the inflammatory pathways leading to NF- $\kappa$ B activation could be blocked by a variety of antioxidants, including N-acetyl-L-cystein, glutathione, thioredoxin, pyrrolidine dithiocarbamate or by over-expression of antioxidant enzymes (Schreck, 1992; McKay *et al.*, 1999). *H. pylori*-induced NF- $\kappa$ B activation and the subsequent up-regulation of cytokines could contribute to inflammatory cell recruitment and retention at sites of infection. Because of its pivotal role in inflammation, NF- $\kappa$ B will be an obvious target for new types of anti-inflammatory treatments for *H. pylori*-induced gastritis (Mori *et al.*, 2000). In the current experiment, long-term administration of rebamipide in *H. pylori* infection significantly attenuated the participation of NF- $\kappa$ B-DNA binding, which resulted in reducing the cytokines involved in *H. pylori*-associated gastric inflammation.

*H. pylori* infection is associated with elevated levels of both mucosal apoptosis and proliferation. The initiation and the regulation of the pathways that promote these paradoxical



**Fig. 8.** Perspective of chemoprevention in *H. pylori*-associated gastric cancer by nimesulide or rebamipide. Retinoic acids or rexinoids are well-known chemopreventive drugs in various cancers, like leukoplakia, breast cancer, and aerodigestive tumors. According to the results in the current study, the long-standing administration of anti-inflammatory drugs like nimesulide or rebamipide could be classified as common chemoprevention since these drugs surely retarded the development of *H. pylori*-associated gastric carcinogenesis in the mice model.

cellular responses are still unclear. However, the any disturbances in maintaining this homeostasis between apoptosis and proliferation could result in definite clinical outcome like peptic ulcer disease or gastric cancer (Asaka, 1998; Moss, 1998; Konturek *et al.*, 2001). Fas Ag, which is a transmembrane receptor, which when bound specifically to its ligand (FasL) trimerizes and initiates a cascade of events resulting in apoptosis. By the way, Fas Ag and Fas L expression had been shown to be regulated at the mRNA level in *H. pylori*-induced gastritis by inflammatory cytokines such as IL-1 $\beta$ , TNF- $\alpha$ , and IFN- $\gamma$  (Jones *et al.*, 1999).

Although there have been several reports showing the increased mucosal inflammatory cytokines, including IL-1 $\beta$ , IL-6, IL-8, TNF- $\alpha$ , and IFN- $\gamma$  in cells or human tissue homogenates of *H. pylori*-associated gastritis (Peek *et al.*, 1995), current study might be the first report revealing the real evidence of the activation of these genes in mouse model of *H. pylori* infection and the attenuation of these cytokines by the long-term administration of anti-inflammatory and radical scavenging drug, rebamipide. According to RNase protection assay of current study, surely *H. pylori* infection increased apoptosis-related genes, caspase-8, Fas Ag, Fas L, FAF, and TRADD, but there were no definite changes of apoptosis-related genes by rebamipide treatment. In mice model of MNU and *H. pylori* administration, rebamipide reduced Fas L and Fas mRNA. We inferred that failure of definite chemopreventive effect of rebamipide could be explained by the maintenance of cell proliferation in spite of long-term rebamipide administration, suggesting the requirement of another apoptosis inducing drug to achieve chemopreventive effect of rebamipide. According to the report by Wang *et al.*

(2000), gastric T cells contribute to apoptosis of the epithelium by a Fas/FasL interaction, which was increasingly expressed during infection with *H. pylori*. In the current experiment, clear decrement of Fas/FasL mRNA was observed in rebamipide administration, but this result was from the total RNA extracted of whole gastric mucosal scratches, not of mucosal T cells.

The natural history of *H. pylori*-associated gastritis is an inexorable progression involving more and more of the stomach, ultimately leading to gastric atrophy (Go, 2000). In general, this process is slow, requiring between 20 and 40 years to complete (Uemura *et al.*, 2001). Atrophic gastritis is widely considered to be a precursor lesion of the intestinal type of gastric cancer (Asaka *et al.*, 1997). A multi-step model for the development of gastric cancer has been proposed beginning with a histological diagnosis of superficial gastritis, which then progressed to chronic atrophic gastritis, followed by intestinal metaplasia, and ultimately, dysplasia (Correa, 1995). In the study shown above, long-term administration of nimesulide or rebamipide resulted in reduced development of chronic atrophic gastritis compared to non-treated group. Although long-term rebamipide administration did not achieve the reduction of gastric carcinogenesis developed by *H. pylori* promoting carcinogenesis of MNU administration, significant reduction in the expression of MDM-2, "big brother" of p53 (Momand *et al.*, 1996), and ODC, accelerator for cell growth (Mori *et al.*, 1996) and significant elevation of pS2/TFF1, possible tumor suppressor protein (Lefebvre *et al.*, 1996), were reached, suggesting the potential implication of rebamipide should be considered as ultimate chemoprevention protocol for gastric carcinogenesis.

## The Perspective of Chemoprevention of *H. pylori*-associated Gastric Carcinogenesis

Many evidences suggest that *H. pylori* surely caused gastric malignancies including adenocarcinoma and lymphoma (Uemura *et al.*, 2001). *H. pylori* provoked acute and chronic gastritis, chronic atrophic gastritis, and gastric adenoma, by which WHO-IARC defined *H. pylori* as class I carcinogen. By the way, since *H. pylori* can be removed by medications composing of one or two weeks of triple drug regimen including proton pump inhibitor and antibiotics, chemoprevention of gastric carcinoma could be possible, if we can definitely confirm the mechanistic links between *H. pylori* infection and gastric cancer. Although our current trial that the long-term administration of anti-inflammatory drugs, nimesulide or rebamipide, showed either the excellent anti-inflammatory effects and partial anti-proliferative effects or definite chemopreventive effects in *H. pylori* infection, but further clinical trials will be required to draw the definite clinical advantage of the longterm administration of antiinflammatory drugs, nimesulide or rebamipide or both (Fig. 8). Molecular biological data surely support the potentiality of longterm administration of nimesulide or rebamipide on chemoprevention. The next step for the chemoprevention of *H. pylori*-associated gastric carcinogenesis will be to prove the concrete clinical advantage of prescribing the anti-inflammatory drugs or eradicating *H. pylori* in the clinical field.

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