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Effect of long-term administration of rebamipide on Helicobacter pylori *infection in mice*

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SUMMARY

Background: It has been suggested that chronic, persistent, uncontrolled inflammations in the stomach could provide the basic step for the beginning of carcinogenesis. One of the potential clinical applications of rebamipide is the inhibition of the immuno-inflammatory response in gastric mucosa imposed by *Helicobacter pylori*.

Aim: To determine the implications of long-term rebamipide treatment in *H. pylori* infection, we studied the underlying moleculo-pathological changes in gastric lesions in mice infected with *H. pylori* (SS1 strain), following this treatment.

Methods: C57BL/6 mice were sacrificed 24 and 50 weeks after *H. pylori* infection, respectively. Colonization rates of *H. pylori*, degree of gastric inflammation and other pathological changes including atrophic gastritis and metaplasia, serum levels of IL-1β, TNF-α, IFN-γ and IL-10, mRNA transcripts of various mouse cytokines and chemokines, and NF-κB binding activities, and finally the presence of gastric adenocarcinoma were compared between an *H. pylori* infected group (HP), and an *H. pylori* infected group administered with long-term rebamipide-containing pellet diets (HPR).

Results: Serum levels of IL-1β, IFN-γ and TNF-α, the gastric mucosal expression of ICAM-1, HCAM and MMP, and transcriptional regulation of NF-κB-DNA binding were all significantly decreased in the HPR group compared with the HP group. An RNase protection assay showed, in the rebamipide administered group, significantly decreased mRNA levels of apoptosis-related genes such as caspase-8, FasL, Fas, TRAIL and various cytokines genes such as IFN-γ, RANTES, TNF-α, TNFR p75, IL-1β. In the experiment designed to provoke gastric cancer through MNU treatment with *H. pylori* infection, the incidence of gastric carcinoma was not different in either group. However, long-term administration of rebamipide showed the advantage of decreasing precancerous lesions like chronic atrophic gastritis and showed molecular evidence of attenuation of proliferation.

Conclusion: The long-term administration of rebamipide should be considered in the treatment of *H. pylori* since it demonstrated molecular and biological advantages like a lessening of gastric inflammation and a possible chemopreventive effect.

INTRODUCTION

The gastric inflammatory response induced by *Helicobacter pylori* consists of neutrophils, lymphocytes, plasma cells and macrophages, along with varying degrees of epithelial cell degeneration and injury.[1] Two possibilities for the mechanism whereby *H. pylori* provokes gastric inflammation have been largely proposed: (i) that *H. pylori* secretes substances that stimulate mucosal inflammation from afar[2] and (ii) through direct contact with gastric epithelial cells and stimulation of cytokine release,[3, 4] which was proven by that fact that

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gastric epithelium from *H. pylori*-infected individuals shows enhanced levels of interleukin-1β (IL-1β), IL-2, IL-6, IL-8, interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α).[5, 6]

Another consequence of persistent inflammation may be alterations in cellular turnover. Gastric epithelial cell proliferation rates within colonized mucosa are significantly increased compared with those in uninfected controls.[7] 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.[8] Although substantial variation exists among apoptosis scores, several studies have reported increased levels of gastric epithelial cell apoptosis among *H. pylori*-infected individuals.[9]

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 the formation of DNA adducts.[10, 11] Following contact with *H. pylori in vitro*, an oxidative burst occurs within polymorphonuclear cells. The specific types of cellular damage which result from the generation of reactive oxygen species include lipid peroxidation, protein oxidation and oxidation of DNA.[12]

Rebamipide has been reported to improve *H. pylori*-associated gastritis either through removing active oxygen species, scavenging of active oxygen species, or by attenuating the production of cytokines or chemokines.[13, 14] Although there have only been several reports which have documented the effects and mechanisms of rebamipide in *H. pylori*-associated gastritis and peptic ulcer disease,[15, 16] they were all carried out over designated periods of administration. No reports have been made on the *in vivo* effects of long-term administration of rebamipide on *H. pylori*-associated gastritis.

Moreover, based on the assumption that chronic, persistent inflammation might predispose to carcinogenesis,[17] the oxidative DNA damage provoked by *H. pylori* infection could be reduced through the eradication of *H. pylori*, and made especially more effective by employing a drug regimen which decreases oxidative stress.[18] Gastric adenocarcinoma can be generated following long-term *H. pylori* infection in Mongolian gerbils, or after the co-administration of *H. pylori* and N-methyl-N-nitrosourea (MNU).[19] This finding could be used to document that: (i) is really long-term rebamipide administration beneficial in attenuating the degree of gastric inflammation? (ii) is really long-term rebamipide administration effective in attenuating halting the progression from chronic gastritis to precancerous or cancerous change? and (iii) what are their fundamental molecular mechanisms? We compared the pathological changes, changes in cellular proteins known to be involved in either inflammation or carcinogenesis, and the mRNA transcription of cytokines, chemokines and apoptosis-related genes among normal controls and in an *H. pylori*-infected group, following the long-term administration of rebamipide. Since we could not observe the development of gastric cancer in a mice model using *H. pylori* infection alone, we added another experiment which included MNU pre-treatment, both with or without additional *H. pylori* infection.

MATERIALS AND METHODS

Mice

A total of 190 specific pathogen free, 6-week-old female C57BL/6 mice (Charles River Japan, Inc.) were housed in steel cages on hard wood chip bedding in an air-conditioned biohazard room with a 12 h L:12 h D cycle. They were given formulated foods and autoclaved distilled water *ad libitum*. The animals were cared for in accordance with institutional guidelines.

Chemicals and bacteria

N-methyl-*N*-nitrosourea (MNU) (Sigma Chemical Co., St Louis, MO) was freshly prepared twice weekly by dissolving it in distilled water at concentrations of 200 p.p.m. The solutions were administered *ad libitum* as drinking water in light-shielded bottles. The mouse-adapted *H. pylori* (SS1, Sydney strain, a kind gift from Prof. Adrian Lee, University of New South Wales, Australia), originally isolated from a peptic ulcer patient, were inoculated on *Brucella* agar plates (Becton Dickinson, Cockysville, MD) containing 10% heat-inactivated foetal bovine serum and Skirrow medium. They were kept at 37 °C under microaerobic conditions using GasPak jars (Difco, Detroit, MI) and CampyPak (Becton Dickinson, Cockeyville, MD). After 24 h of fasting, a 0.1 mL suspension of *H. pylori* containing 1×10^8 colony-forming units (CFU)/mL was delivered intragastrically to the mice using an oral catheter. Rebamipide was administered mixed in chow pellet diets. According to preliminary data from the Otsuka

Pharmaceutical Research Center (Tokushima, Japan), treatment of 20 mg/kg/day rebamipide maintained a 0.02% blood concentration of the drug. In order to maintain the desired blood levels of the drug, the amounts of pellet diet containing rebamipide consumed were monitored daily.

Experimental design (Figure 1)

Out of a total of 190 C57BL/6 mice, 150 mice were divided into five groups of 30 (Group 3 and Group 6 were allocated 40 mice per group due to a low *H. pylori* colonization rate) and were given MNU mixed in their drinking water at concentrations of 200 p.p.m. (Groups 4, 5 and 6) for total five cycles of a 1-week regimen with a 1-week pause. After completion of MNU administration, they were given autoclaved distilled water for 2 weeks, and Groups 4 and 6 were further inoculated with *H. pylori* three times every other day. *H. pylori* was given to 20 mice without prior MNU treatment in Groups 2 and 3 and the remaining 20 mice (Group 1), as noncarcinogen controls, were only given distilled water without *H. pylori* or MNU. At 24 weeks after *H. pylori* administration, 10 mice from each group were sacrificed to look for the presence of *H. pylori* by direct culture and CLO-test. Serology tests were also performed to check IgG anti-*H. pylori* antibody levels. The observation period was then extended to 50 weeks after *H. pylori* infection.

Histopathological examination

All animals were sacrificed under deep ether anaesthesia at the 24th or 50th week after *H. pylori* infection. The stomachs, which were 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 μm for haematoxylin and eosin (H&E) staining. Histological typing was based on the predominant pattern of the tumour and the lesions of the glandular stomach were classified by the following criteria. Tubular adenocarcinomas were defined as tumour 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 tumour cells containing large amounts of mucins. Adenoma was defined as a proliferative immature glandular epithelium with varying degrees of dysplasia, and foveolar hyperplasia was defined as an increased height of the pit with hyperchormatic nuclei and mitotic

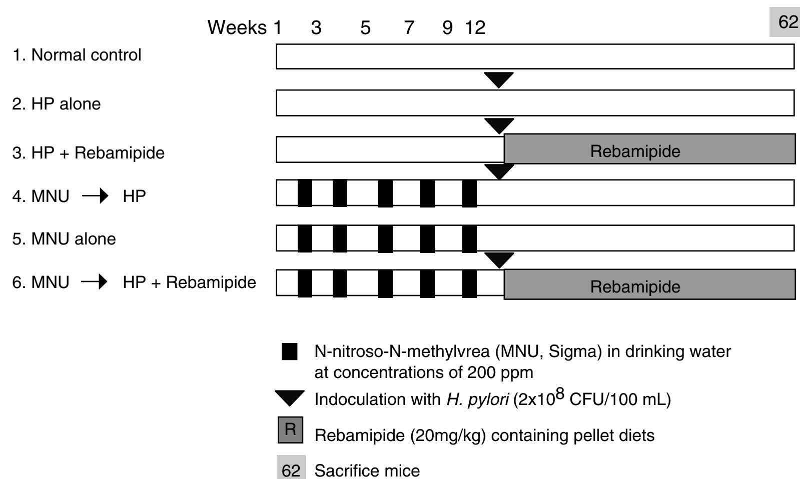

Figure 1. Study protocol. Of a total of 190 C57BL/6 mice, 150 were divided into five groups of 30 mice in each group (Groups 2 and 4 were allocated 40 mice per group due to the relatively low colonization rate of *H. pylori* compared with the other groups) and were given MNU mixed in their drinking water at concentrations of 200 p.p.m. (Groups 4, 5 and 6) for a total of five cycles of a 1-week regimen with a 1-week pause. After completion of MNU administration, they were given autoclaved distilled water for 2 weeks, and groups 4 and 6 were further administered with cultured *H. pylori* three times every other day. *H. pylori* was given to 20 mice without prior MNU treatment (groups 2 and 3), and the remaining 20 mice were given only distilled water without either *H. pylori* or MNU as noncarcinogen controls (Group 1).

activity. The presence of intestinal metaplasia and gastric atrophy was also measured. Atrophy of the gastric mucosa was defined as a loss of glandular tissue. Atrophy leads to thinning of the mucosa. Loss of glands may follow erosions or ulceration of the mucosa with destruction of the glandular layer, or result from a prolonged inflammatory process.

Confirmation of the presence of H. pylori *in gastric mucosa*

In order to confirm *H. pylori* infection, 30 mm^2 samples of stomach mucosa from the greater curvature containing both fundic and pyloric glands were transferred to 1.0 mL of sterile 0.1 mol/L PBS, homogenized, and plated on selective trypticase soy agar/5% sheep blood plates containing vancomycin (20 mg/mL), nalidixic acid (10 mg/mL), bacitracin (30 mg/mL), and amphotericin B (2 mg/mL) (Sigma Chemical Co., St Louis, MO), and grown for 3–5 days. Colonies were identified as *H. pylori* based on their resistance to the antibiotics listed above, characteristic morphology by Gram stain, and by urease, catalase and oxidase activity. Another 3 × 3 mm sample from antrum was put into the gel of a rapid urease test kit (CLO-test, Delta West, Australia) and left for 6 h at room temperature to test for urease activity. If the colour of the gel changed from yellow to red we considered the test positive. Presence of *H. pylori* in the gastric pit was further confirmed by Warthin–Starry silver staining.

Western blot analysis

Gastric mucosal scratches from five mice per group were mixed together and homogenized in iced cold RIPA buffer (1 × PBS/1% Nonidet P-40/0.5% sodium deoxycholate/0.1% SDS at 10 mg/mL). After fractionating in 4–12.5% SDS/PAGE, the proteins were transferred to nitrocellulose filters. The filters were probed with each antibody (Santa Cruz Biotechnology, Santa Cruz, CA), developed using the ECL chemiluminescence system, and exposed to Kodak XAR5 film (Eastman-Kodak, Rochster, NY). The antibody types used for the current experiment were all purchased from Santa Cruz Biotechnology, except for pS2/TFF1 antibody, which was 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-β1 (TGF-β1); an anti-inflammatory cytokine, heat shock protein 70 (Hsp 70), MDM-2; a regulatory gene of p53, proliferating cell nuclear antigen (PCNA), Bcl-2, Bax, cyclo-oxygenase-2 (COX-2), *h-met*; a receptor molecule for hepatocyte growth factor, ornithine decarboxylase (ODC); a rate-limiting enzyme for polyamine synthesis.

Zymography for matrix metalloprotease (MMP)

Gastric tissues were homogenized, mixed with sample buffer, and directly applied, without prior heating or reduction, to 4–16% acrylamide gels containing 1 mg/mL of gelatin. After removal of SDS from the gel by incubation in 2.5% (v/v) Triton X-100 for 1 h, the gels were incubated at 37 °C for 16 h in 50 mM Tris-HCl, pH 7.6, containing 0.2 M NaCl, 5 mM $CaCl_2$ and 0.02% (w/v) Brij-35. The gels were stained for 3 h in 40% methanol/10% glacial acetic acid containing 0.2% Coomassie Brilliant Blue G-250 and de-stained in the same solution without dye. The activity of each MMP was evident as a clear band against the blue background of stained gelatin.

Electrophoretic mobility shift assay (EMSA) for transcription factor

Nuclear proteins were extracted from the mucosal homogenates from each group. Three mice were randomly selected from each group and their mucosal homogenates were scraped on to iced plates to obtain the mucosal nuclear proteins. Nuclear proteins (10 μg) were incubated for 30 min at 25 °C with 20 pg of ^{32}P-labelled oligonucleotides containing the NF-κB binding site (GATCGAGGGGACTTTCCCAGC) and 1 μg of poly dI-dC in 5 μL of a solution consisting of 20 mM HEPES, 4 mM $MgCl_2$, 50 mM $CaCl_2$, 1 mM EDTA, 1 mM DTT and 4% glycerol. The mixtures were loaded on to a nondenaturing 6% polyacrylamide gel with 0.25× TBE electrophoresis buffer. After electrophoresis, the gels were dried and exposed to the radiography film for 24 h at − 70 °C with intensifying screens. Using nuclear proteins from Group 2, EMSA was done for AP-1, Sp-1 and CREB to determine the involvement of these transcription factors in *H. pylori*-associated gastritis as well.

Determination of IL-1β, IL-10, IFN-γ and TNF-α

Sera were obtained during the sacrifice of mice. All cytokine levels of mouse serum IL-1β, IL-10, IFN-γ and

TNF-α were measured by ELISA kit (R&D, Mineapolis, MN) according to manufacturer's instructions. These measurements were made in triplicate and repeated in three separate experiments.

Ribonuclease protection assay (RPA)

The RPA is a highly sensitive and specific method for the detection and quantification of mRNA species. This assay was made possible by the discovery and characterization of DNA-dependent RNA polymerase from the bacteriophages SP6, T7 and Ts, and the discovery of their cognate promoter sequence. The probe used for the RNase protection assay was synthesized using a protocol provided with the RiboQuant *in vitro* transcription kit (PharMingen, San Diego, CA), using 100 mCi of 800 Ci/mmol [α-^{32}P] UTP (Amersham Pharmacia Biotech, UK) and probes purchased from PharMingen, in the multiprobe RNase protection assay system. We used mCK-2 containing interleukin-12p35 (IL-12p35), IL-12p40, IL-10, IL-1α, IL-1β, monokine induced by IFN-γ (MIG), IL-6, and interferon-γ (IFN-γ), mCK-3 containing tumour necrosis factor-β (TNF-β), lymphotoxin-β (LT-β), TNF-α, IL-6, IFN-γ, IFN-β, transforming growth factor-β1 (TGF-β1), TGF-β2, mCK-5 containing lymphotactin (Ltn), regulated on activation, normal T-cell expressed and secreted (RANTES), Eotaxin, macrophage inflammatory protein-1β (MIP-1β), MIP-1α, MIP-2, IFN-γ 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), and receptor-interacting protein (RIP). Total RNA was extracted from five mouse gastric mucosa layers from each group, for which the mice were selected randomly. The excised mucosal layers were kept in 'RNA later' solution (Ambion) to avoid activating the RNase. 20 μg of total RNA were hybridized at 42 °C overnight and ribonuclease protection assays were performed according to the manufacturer's protocol. The protected fragments were analysed together with a sequencing reaction on a 5% polyacrylamide gel containing 8 M urea. The gels were fixed, dried and exposed to film.

Statistical analysis

An unpaired Student's *t*-test with ANOVA analysis was used for a comparison of the data and a *P*-value of <0.05 was considered statistically significant.

RESULTS

Effects of the long-term administration of rebamipide on bacterial colonization

A preliminary study demonstrated that all the mice from Group 2 (*H. pylori* alone) and Group 4 (MNU + *H. pylori*) at the 24 weeks after *H. pylori* infection showed positive colonization, which was proven by direct bacterial culture and rapid urease test, but only 65% of them showed positive *H. pylori* colonization in the rebamipide administration group. In addition, although we were able to recover 2.23 (± 0.12) × 10^5 CFU *H. pylori* per μg of gastric tissue from mice orally inoculated with *H. pylori* SS1, the rebamipide administered group showed only 1.15 (±0.23) × 10^5 CFU *H. pylori* per μg of gastric tissue. Warthin–Starry stain from all animals inoculated with *H. pylori* infected gastric cells showed numerous spiral bacteria along the length of the gastric pits in both the antrum and the body. Based on these preliminary data, we increased the numbers of mice to 30 mice for Groups 3 and 6, as well as increasing the bacterial loads to 1 × 10^8 CFU per 100 μL for these groups.

Effect of long-term administration of rebamipide on tumour incidence in mice

The survival rate of each group was more than 85%, and all the animals in Group 3 survived until the 50th week (Table 1). The incidence of gastric tumours at the 50th week was 64.7%, 10.5% and 61.1% in Groups 4, 5 and 6, respectively, suggesting a significantly higher incidence of gastric tumours in mice treated with the combination of MNU and *H. pylori* infection. These results signified that *H. pylori* infection surely promoted MNU-induced gastric carcinogenesis. The groups with *H. pylori* alone (Group 2) or *H. pylori* administered with long-term rebamipide (Group 3) did not develop any gastric tumours at 50 weeks. Following a pathological evaluation of the gastric tumours which developed in Groups 4–6, gastric adenocarcinoma was noted in 41.2% of Group 4, 5.3% of Group 5 and 33.3% of

Table 1. Comparison of tumour development according to group

Treatment group	Total no. of mice	Effective no. of mice	No. of dead mice (%)	No. of tumour bearing mice (incidence)
2. HP alone	20	19	1 (5)	0
3. HP + Rebamipide	30	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	30	18	2 (10)	11 (61.1)

Treatment group	Effective no. of mice	No. of tumour bearing mice	Adenoma (%)	Adenocarcinoma (%)
4. MNU → HP	17	11	4 (23.5)	7 (41.2)
5. MNU alone	19	2	1 (5.3)	1 (5.3)
6. MNU → HP + Rebamipide	18	11	5 (27.8)	6 (33.3)

Group 6. No significant findings except chronic atrophic gastritis and foveolar hyperplasia were observed in Group 2 (50th week). This finding implies that *H. pylori* infection might play a role as promoter in gastric carcinogenesis rather than as a 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 the adenocarcinoma was tubular, and the remaining one-third was signet ring cell carcinoma. Most of the tumours (18/23) were found in the antrum.

In contrast to the lack of 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 (Table 2). In these mice, marked infiltrations of lymphocyte, plasma cells and neutrophils, and eosinophils (at a lower frequency) were present in the lamina propria and submucosa and formations of lymphoid follicles. Interestingly, Group 6 (MNU + *H. pylori* with long-term rebamipide administration) showed a markedly low incidence of atrophic gastritis compared with Group 4 (MNU + *H. pylori*), suggesting that long-term rebamipide administration significantly reduced the development of chronic atrophic gastritis.

Effects of long-term administration of rebamipide on the expression of various gastric proteins

We divided the various proteins into two groups based on their implied method of action: inflammation related (Figure 2A) or cell growth related (Figure 2B). After *H. pylori* infection, ICAM-1, HCAM (CD44), MMP, COX-2 and Hsp 70 were definitely induced or increased. On the other hand, long-term administration of rebamipide mixed in the pellet diets caused an attenuation in the expression of ICAM-1, HCAM and MMP, and increased the expression of Hsp 70. These results implied that rebamipide was acting to either decrease the proteins involved in the propagation and aggravation of gastric inflammation, or to increase cytoprotective proteins. TGF-β1, a well-known anti-inflammatory cytokine, was slightly increasingly expressed in Group 6 (MNU + *H. pylori* + rebamipide). COX-2 expression was not prominent, irrespective of rebamipide or *H. pylori*, but significantly increased in the MNU + *H. pylori* group. In

Table 2. Incidence of pre-cancerous lesions of mice (50 weeks); intestinal metaplasia and/or adenoma

Group treatment	No. of mice observed	Atrophic gastritis	Intestinal metaplasia	Gastric adenoma
2. HP alone	19	13 (68.4)	1 (5.3)	0
3. HP + Rebamipide	20	14 (70.0)	0	0
4. MNU + HP	17	12 (70.6)	4 (23.5)	4 (23.5)
5. MNU + alone	19	8 (42.1)	4 (21.1)	1 (5.3)
6. MNU HP + Rebamipide	18	4 (22.2)*	2 (11.1)	5 (27.8)

*Group 4 vs. Group 6 ($P < 0.05$).

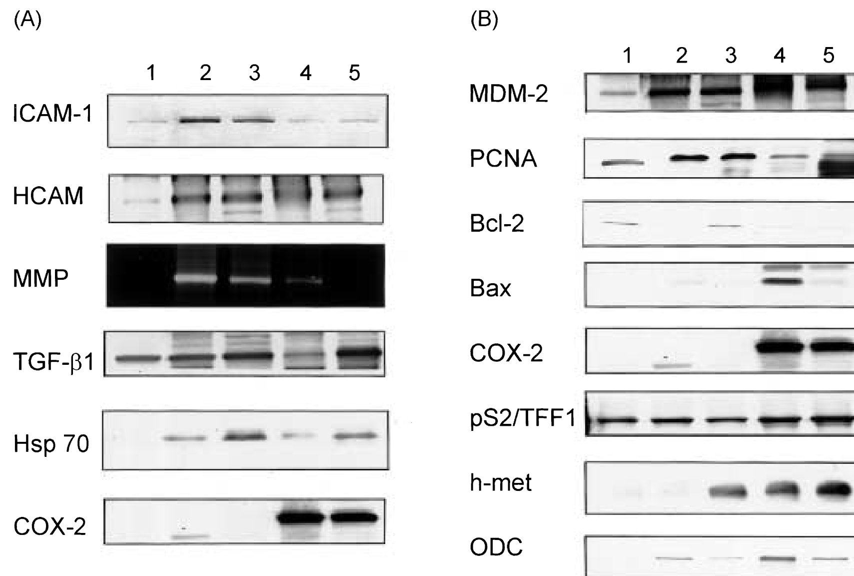

Figure 2. Changes in the protein profiles involved in (A) inflammation and (B) tumorigenesis. (A) Compared with normal controls (lane 1), the *H. pylori*-infected stomach (lane 2) showed increased expressions of ICAM-1, HCAM, MMP and Hsp70. Lane 3 denotes *H. pylori* infected mice administered with long-term rebamipide, and showing marked attenuation of ICAM-1 and MMP and increased expressions of TGF-β1 and Hsp 70 than lane 2, signifying that the long-term administration of rebamipide decreased proteins involved in the propagation or amplification of inflammation. (B) *H. pylori* infection increased the expression of MDM-2, PCNA and ODC. Compared with lane 4 (MNU + *H. pylori*), lane 5 (MNU + *H. pylori* with long-term administration of rebamipide) showed a decreased expression of MDM-2, PCNA, Bax and ODC. On the other hand, the expression of pS2/TFF1 and *h-met* was significantly increased in lane 5 compared with 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. The proteins used for the current experiment were extracted from gastric mucosal scratches of five mice per lane.

Group 4 (MNU + *H. pylori*), ornithine decarboxylase (ODC), Bax, pS2/TFF1, h-met and COX-2 were all significantly increasingly induced, suggesting increased cellular proliferation, but the levels of expression of ODC, COX-2 and PCNA were significantly decreased after further chronic administration of rebamipide. pS2/TFF1, a tumour suppressor protein which is particularly involved in gastric carcinogenesis, was slightly increased after the long-term administration of rebamipide.

As indicated by an ELISA of cytokines such as IFN-γ, TNF-α, IL-1β and IL-10 (Figure 3), *H. pylori* infection increased all the above cytokines. Long-term administration of rebamipide significantly decreased IFN-γ and TNF-α ($P < 0.05$). Although rebamipide decreased IL-1β and increased IL-10, neither showed any statistical significance.

Effects of the long-term administration of rebamipide on NF-κB-DNA binding

Figure 4 shows the NF-κB complex of nuclear proteins extracted from each group detected by electrophoretic mobility shift assay. The nuclear proteins were extracted from three randomly selected mice stomachs from each group, suggesting the EMSA band as the mean activity of each group. *H. pylori* infection (lane 2) increased NF-κB-DNA binding significantly over the uninfected group (lane 1). Rebamipide treatment significantly decreased NF-κB binding (16.6% of lane 2). However, administration of MNU only caused slight a increment in NF-κB binding, irrespective of *H. pylori* infection, but lane 6, from the group treated with rebamipide, also attenuated the NF-κB binding than MNU and *H. pylori* group (lane 4), suggesting a possible action of rebamipide in modulating the inflammatory response through the transcriptional regulation of NF-κB.

Effects of long-term administration of rebamipide on the mRNA of cytokine and apoptosis-related genes

We investigated the various mRNA transcripts of mouse cytokines and chemokines by multiprobe RNase protection assay. Figure 5A shows the ribonuclease protection assay of mCK-3 (RiboQuant, multiprobe RPA) giving

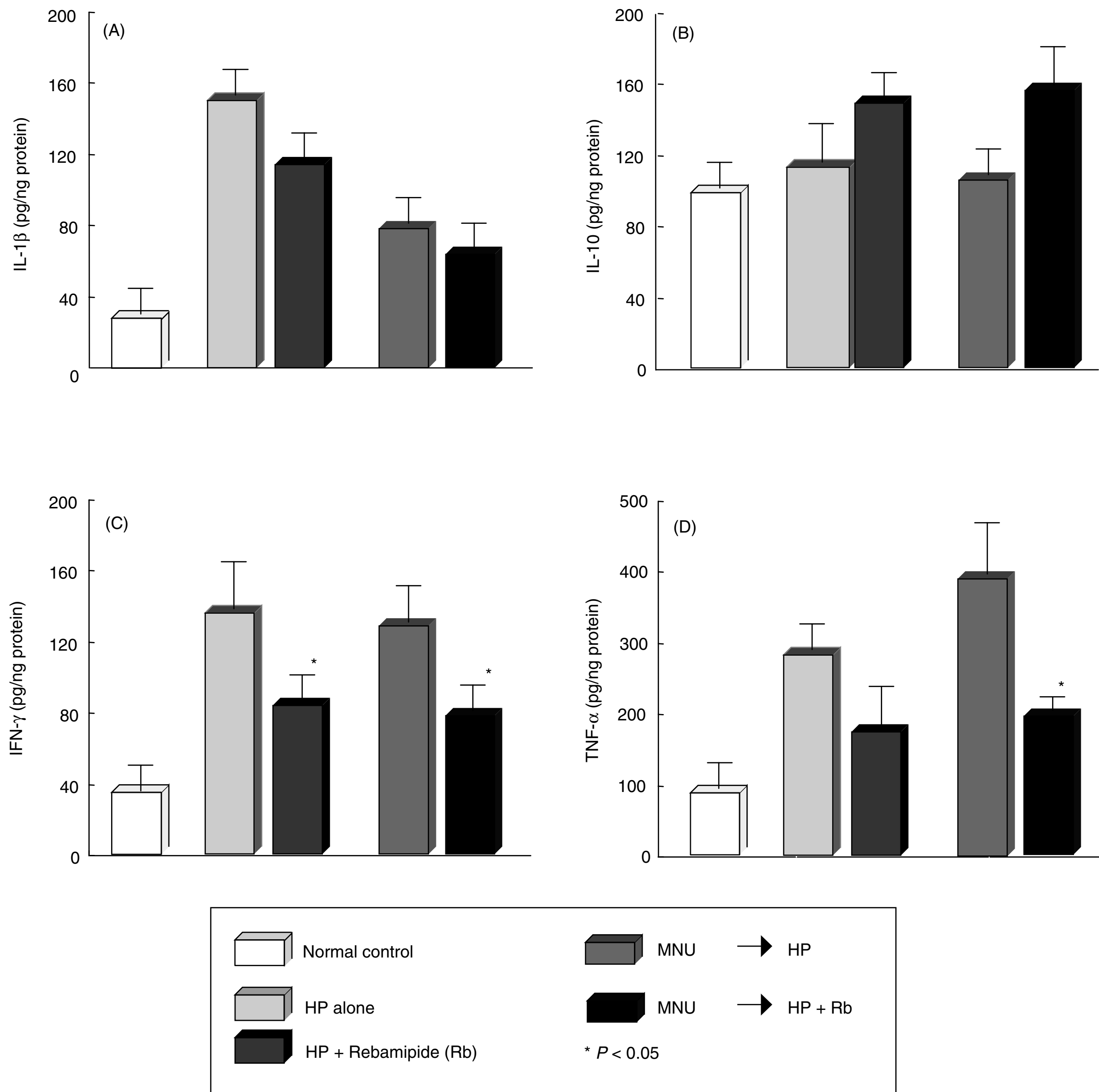

Figure 3. Changes in serum cytokines levels according to group. Changes in (A) IL-1β, (B) IL-10, (C) IFN-γ and (D) TNF-α. *H. pylori* infection increased all the cytokines levels of IL-1β, IFN-γ and TNF-α, except for IL-10. Long-term administration of rebamipide significantly decreased IFN-γ and TNF-α ($P < 0.05$). Although rebamipide decreased IL-1β and increased IL-10, these results did not reach statistical significance.

TNF-β, LTβ, TNF-α, IL-6, IFN-γ, IFN-β, TGF-β1 and TGF-β2. Among these, TNF-β, LTβ, TNF-α, IL-6 and IFN-γ were increased after chronic *H. pylori* infection. After the long-term administration of rebamipide, all of these transcripts were significantly decreased (lane 2 vs. lane 3). No significant change was noted in the MNU-treated group irrespective of rebamipide administration. Figure 5B shows the ribonuclease protection assay of mCK-5 exhibiting chemokines like Ltn, RANTES, Eotaxin, MIP-1β, MIP-1α, MIP-2, IP-10, MCP-1 and TCA-3. Similar to the cytokines, *H. pylori* infection increased all of these chemokines except for MIP-2 and TCA-3. The long-term administration of rebamipide showed marked decreases in the transcript levels of RANTES, Lin and IP-10 in the MNU treated group, as well as decreasing RANTES, eotaxin and MIP-1α. Figure 6A, B also shows the ribonuclease protection assay of cytokines and their receptor mRNA, revealing

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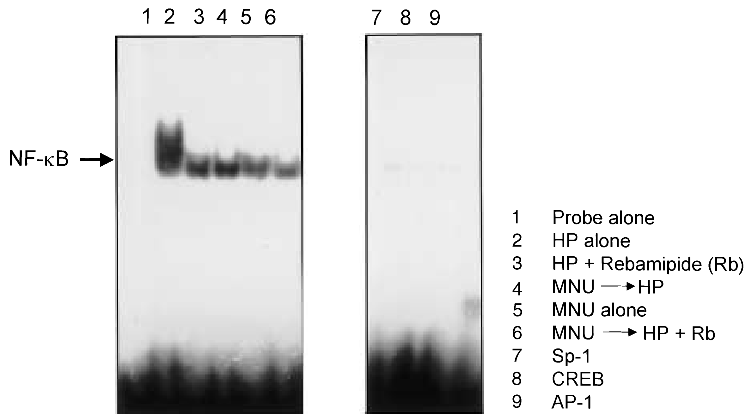

Figure 4. Effects of long-term rebamipide on NF-κB binding EMSA of the NF-κB, Sp-1, CREB and AP-1 showing increased DNA binding after *H. pylori* infection than uninfected mice (lane 1). The nuclear proteins of each lane were extracted from the three randomly chosen mice stomachs of each group, suggesting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

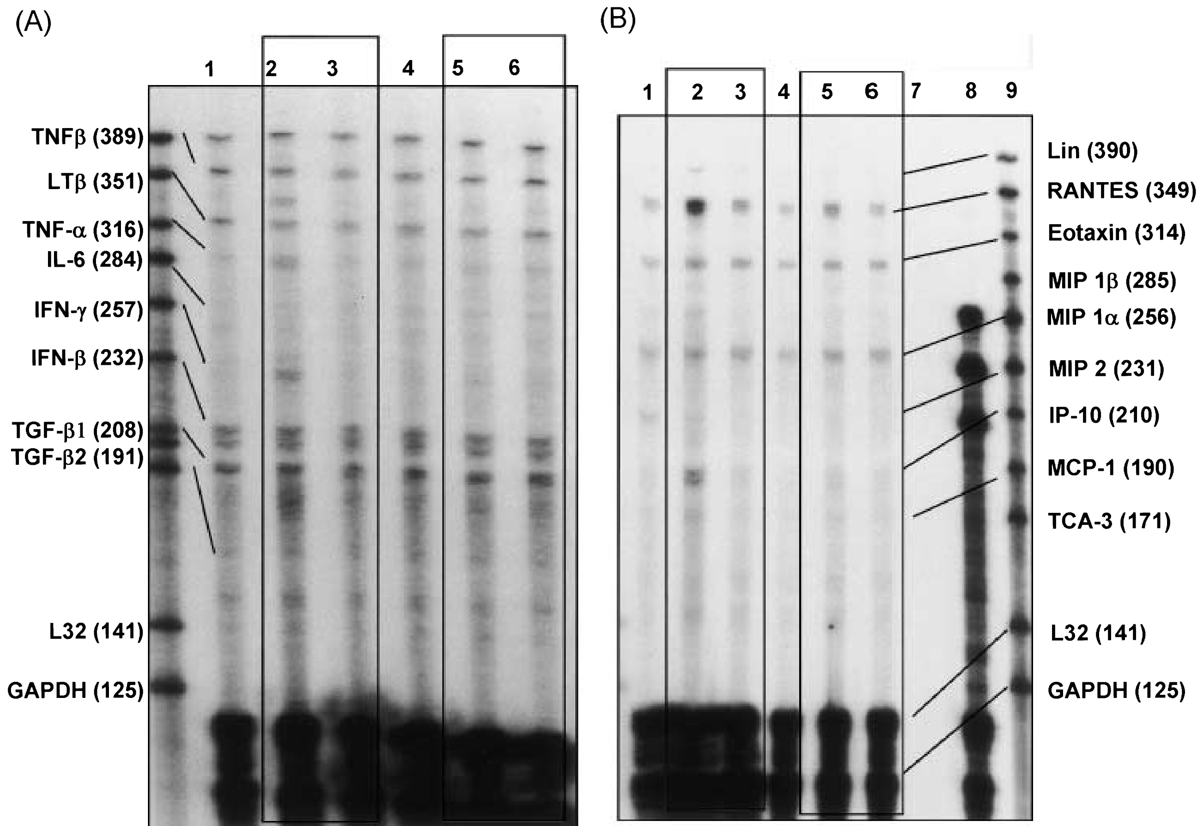

Figure 5. Effects of long-term rebamipide treatment on the mRNA of cytokines genes. (A) Multi-probe ribonuclease protection assay of mouse cytokines [mCK-3 kit (RiboQuant, PharMingen cat no. 45003p)] contains TNF-β, LTβ, TNF-α, IL-6, IFN-γ, IFN-β, TGF-β1 and TGF-β2. Among them, TNF-β, LTβ, TNF-α, IL-6 and IFN-γ were increased after chronic *H. pylori* infection, which were significantly decreased after long-term administration of rebamipide. (B) Multi-probe ribonuclease protection assay of mouse cytokines [mCK-5 kit (RiboQuant, cat no. 45026p)] ribonuclease protection assay of mCK-5 displayed chemokines such as Ltn, RANTES, Eotaxin, MIP-1β, MIP-1α, MIP-2, IP-10, MCP-1 and TCA-3. Similar to (A), *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 mRNA 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.

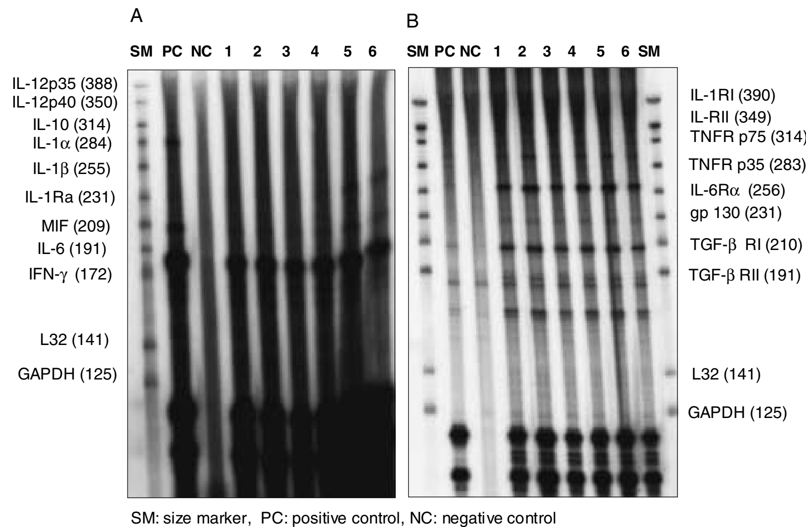

Figure 6. Effects of the long-term administration of rebamipide on the mRNA of cytokines and cytokine receptor genes. (A) [ribonuclease protection assay of mCK-2 (RiboQuant, cat no. 45002p)] and (B) [ribonuclease protection assay of mCR-4 (RiboQuant, cat no. 45364p)] showed the ribonuclease protection assay of cytokines and their receptor mRNA, showing the attenuating effects of long-term rebamipide on mRNA of IL-1Rα, TNFR and gp130. 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.

the attenuating effects of long-term administration of rebamipide on IL-1Ra, TNFR and gp130. Figure 7 showed the ribonuclease protection assay of mAPO-3 containing caspase-8, FasL, Fas, FADD, FAP, FAF, TRAIL, TNFRp55, TRADD and RIP. *H. pylori* infection increased the genes involved in apoptosis. Slight decreases in the transcription of caspase-8, FasL, Fas and TRAIL were observed following long-term administration of rebamipide pre-treated with MNU.

DISCUSSION

This study showed that rebamipide, an anti-ulcer and anti-inflammatory drug, demonstrated significant attenuating effects in the severity of *H. pylori*-associated gastritis, based on the finding that rebamipide decreased levels of the cell adhesion molecules ICAM-1 and HCAM, reduced MMP activities, decreased COX-2 expression, reduced the DNA binding of the inflammation-associated transcription factor NF-κB, and decreased the activity of cytokines such as IL-1β, TNF-α, RANTES, IP-10, MIP-1α and IFN-γ. However, although it has been hypothesized that chronic, persistent inflammation might predispose to carcinogenesis, the long-term administration of rebamipide did not show definite evidence of chemoprevention despite an excellent anti-inflammatory action. However, the latter finding 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 effective chemoprevention.[20] However, the development of pre-cancerous lesions as in chronic atrophic gastritis was surely retarded by its long-term administration. Due to a lack of complete anti-proliferation and induction of apoptosis, rebamipide requires supplementation by another chemopreventive modality to match the hypothesis. Based on this finding, we can define rebamipide as common chemopreventive agent, since retinoic acid, tamoxifen and nonsteroidal anti-inflammatory drugs (NSAIDs) could be specified as specific chemopreventive agents.

The current study might well be the first to show the effect of long-term administration of rebamipide in an *H. pylori*-infected animal model. Before this, a rat model had been demonstrated with chronic gastritis which was developed by the continuous administration of 5 mM sodium taurocholate for 6 months. Rebamipide treatment for 4 weeks (6 and 60 mg/kg/day) initiated after development of chronic gastritis, dose-dependently attenuated the gastritis in terms of degree of inflammatory cell infiltration, erosion and glandular atrophy.[21] The proposed mechanisms for the anti-inflammatory and mucosal protective actions of rebamipide were decreased neutrophilic activitity,[22] decreased production

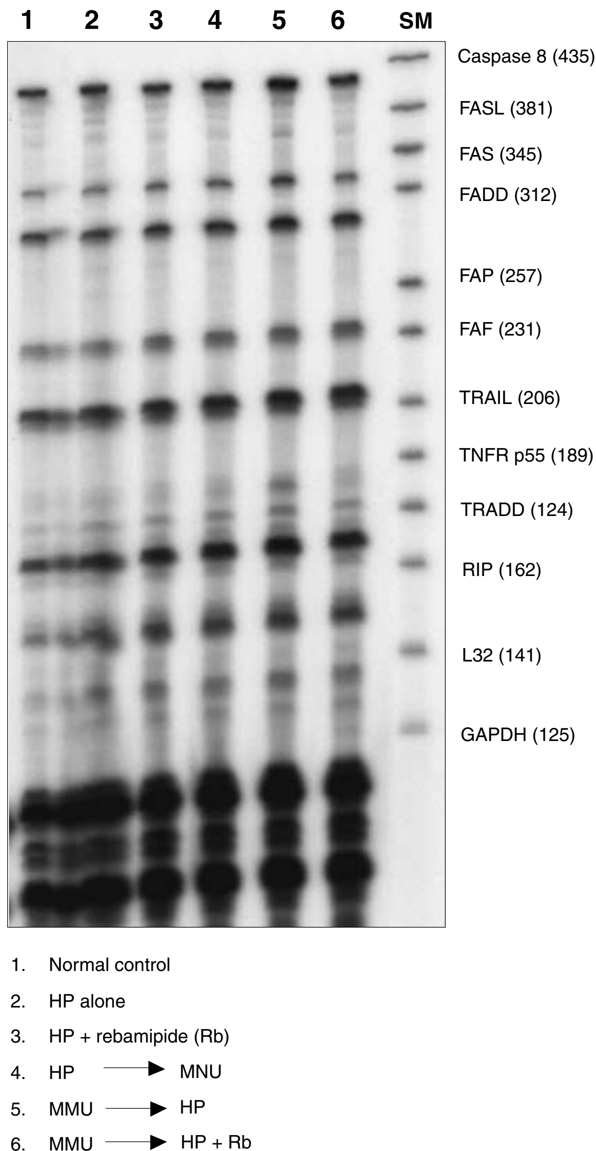

Figure 7. Effects of the long-term administration of rebamipide on mRNA of apoptosis related genes. Ribonuclease protection assay of mAPO-3 (RiboQuant, cat no. 45355p) dealt with caspase-8, FasL, Fas, FADD, FAP, FAF, TRAIL, TNFRp55, TRADD and RIP. *H. pylori* infection increased the genes involved in apoptosis. Slight decreases in transcript of caspase-8, FasL, Fas and TRAIL were observed in long-term administration of rebamipide pre-treated with MNU.

of inflammatory cytokines[23] and a decreased generation of oxygen free-radicals.[24]

Since *H. pylori* generally adheres to gastric epithelial cells without invading the epithelium, it is presumed that an interaction between the bacteria and host epithelia cells may initiate an *H. pylori*-induced inflammation.[25] Due to the strong inflammatory response to *H. pylori* infection, the role of inflammatory cytokines has been investigated and it has been shown that mucosal biopsy specimens from patients with *H. pylori* infection contain significantly elevated levels of IL-1β, IL-6, TNF-α and IL-8 compared with those in specimens from uninfected individuals.[26–28] Most intracellular bacteria generally induce Th1 responses, while extracellular pathogens stimulate Th2 responses. Based on the fact that *H. pylori* is noninvasive 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-γ than IL-4 or IL-10, which is reflective of a Th1-type response.[29] Therefore, certain strains of mice (C57BL/6) infected with *H. felis* that develop a Th1-type 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.[30] The adoptive transfer of Th2 lymphocytes from infected mice into infected recipients reduces bacterial colonization density, while the transfer of Th1 cells increases the severity of gastritis.[31] In the current experiment, we were able to confirm that a definite Th1-type response was generated by *H. pylori* infection and that long-term administration of rebamipide, an anti-inflammatory drug, attenuated Th1-type cytokine generation and increased Th2-type cytokines, which led to a decreased gastric inflammation.

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. The expression of cell adhesion molecules on gastric epithelial cells may participate in leukocyte homing and epithelial cell adhesions in *H. pylori*-associated mucosal inflammations.[32] 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 the up-regulation of steady-state mRNA levels and the cell

surface expressions of ICAM-1. *H. pylori* activated the ICAM-1 or IL-8 promoter via the NF-κB binding site.[33] In the current study, the 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-κB has important implications, since other NF-κB responsive genes, including those encoding IL-8, TNF-α, IL-1 and IL-6, have been found to be elevated in the gastric mucosa of people infected with *H. pylori*.[23–34] NF-κB 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-κB activation could be blocked by a variety of anti-oxidants, including N-acetyl-L-cystein, glutathione, thioredoxin and pyrrolidine dithiocarbamate, or by the over-expression of anti-oxidant enzymes.[35, 36] *H. pylori*-induced NF-κB 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-κB will be an obvious target for new types of anti-inflammatory treatments for *H. pylori*-induced gastritis.[32] In the current experiment, the long-term administration of rebamipide in *H. pylori* infection significantly attenuated the participation of NF-κB-DNA binding, which resulted in a reduction in the production of 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 regulation of the pathways that promote these paradoxical cellular responses are still unclear. However, the any disturbances in the maintenance of this homeostasis between apoptosis and proliferation could result in a definite clinical outcome such as peptic ulcer disease or gastric cancer.[20, 37, 38] Fas, a transmembrane receptor, which when bound specifically to its ligand (FasL), trimerizes and initiates a cascade of events resulting in apoptosis. Incidentally, 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-1β, TNF-α and IFN-γ.[39]

Although there have been several reports showing increased mucosal inflammatory cytokines, including IL-1β, IL-6, IL-8, TNF-α and IFN-γ in cells or human tissue homogenates of *H. pylori*-associated gastritis,[40] current study might be the first report to reveal the real evidence of the activation of these genes in a 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 the ribonuclease protection assay of current study, *H. pylori* infection surely increased levels of the apoptosis-related genes, caspase-8, FasAg, FasL, FAF and TRADD, but there were no definite changes in apoptosis-related genes following rebamipide treatment. In the mouse model of MNU and *H. pylori* administration, rebamipide reduced FasL and Fas mRNA. We inferred that the failure of a definite chemopreventive effect of rebamipide could be explained by the maintenance of cell proliferation despite long-term rebamipide administration, suggesting the requirement for another apoptosis-inducing drug to achieve a chemopreventive effect of rebamipide. According to the report by Wang *et al.*,[41] gastric T cells contribute to apoptosis of the epithelium via a Fas/FasL interaction, which was increasingly expressed during infection with *H. pylori*. In the current experiment, a clear decrement of Fas/FasL mRNA was observed in rebamipide administration, but this result came from the total RNA extracted from whole gastric mucosal scratches, not from 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.[42] In general, this process is slow, requiring between 20 and 40 years to complete.[43] Atrophic gastritis is widely thought to be a precursor lesion of the intestinal type of gastric cancer.[44] A multistep model for the development of gastric cancer has been proposed, beginning with a histological diagnosis of superficial gastritis, which then progresses to chronic atrophic gastritis, followed by intestinal metaplasia and ultimately dysplasia.[45] In the current study, the long-term administration of rebamipide resulted in a reduced development of chronic atrophic gastritis compared with an untreated group. A previous study also showed that eradication rates of *H. pylori* were significantly increased in groups supplemented with an anti-oxidant including rebamipide or other ascorbic acid. Although long-term rebamipide administration did not achieve the reduction of gastric carcinogenesis developed by *H. pylori*'s promoting carcinogenesis of MNU administration, significant reductions in the expression of MDM-2, 'big brother' of p53[46] and ODC, accelerator for cell growth[47] and a significant elevation in pS2/TFF1, a possible tumour suppressor protein[48] were achieved, suggesting that rebamipide

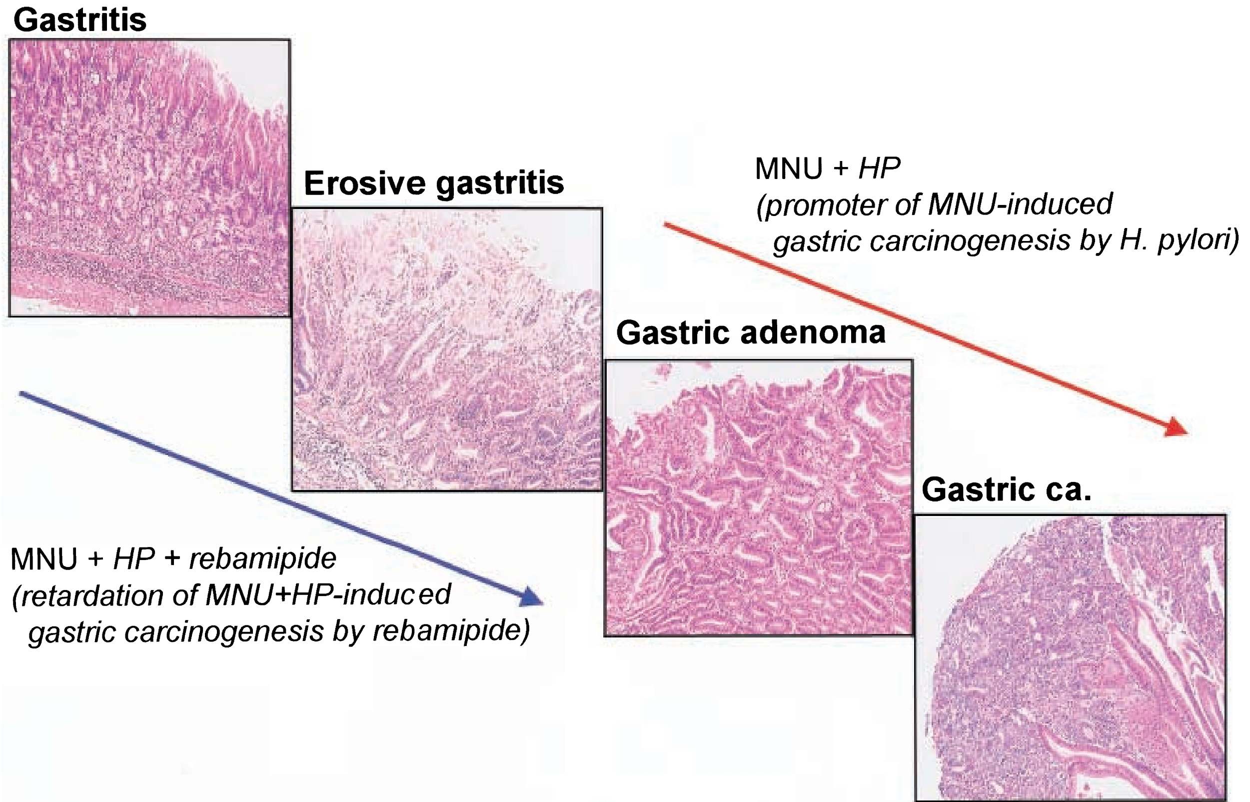

Figure 8. Histopathologies of erosive gastritis, gastric adenoma, and gastric adenocarcinoma after MNU and/or *H. pylori* infection. The representational histological features observed: (A) erosive gastritis after *H. pylori* infection, (B) gastric adenoma after MNU and *H. pylori* infection, and (C) gastric adenocarcinoma.

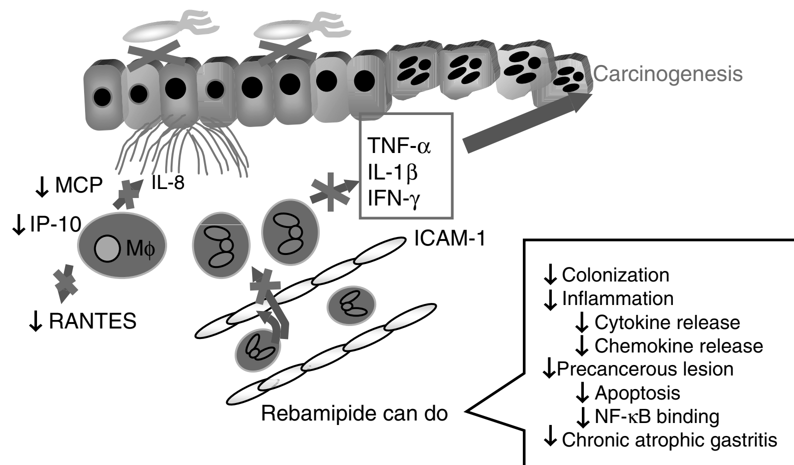

Figure 9. Schematic presentation showing the effects of the long-term administration of rebamipide on *H. pylori* infection. In the early stages of *H. pylori* infection, rebamipide hindered the colonization and adherence to gastric mucosal cells. Long-term administration of rebamipide decreased several cytokines and chemokines, which were important in the perpetuation and worsening of *H. pylori*-associated gastric inflammation. Some genes known to be involved in gastric carcinogenesis could be attenuated by the long-term administrations of rebamipide.

could be possibly be considered as an ultimate chemoprevention protocol for *H. pylori*-associated gastric carcinogenesis.

In conclusion, the long-term administration of rebamipide showed excellent anti-inflammatory effects and partial anti-proliferative effects in long-standing *H. pylori* infection (Figures 8 and 9), but further evaluation will be required to draw real clinical implications of long-term rebamipide administration.

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