Inhibitory Effect of Rebamipide on the Neutrophil Adherence Stimulated by Conditioned Media from *Helicobacter pylori*-Infected Gastric Epithelial Cells

CHI DAE KIM, HYUN HEE KIM and KI WHAN HONG

**ABSTRACT**

We investigated the mechanism or mechanisms by which rebamipide protects against the gastric mucosal inflammation associated with *Helicobacter pylori*. The production of interleukin (IL)-8 in association with expression of IL-8 mRNA was greatly increased in the *H. pylori*-infected Kato III cells in a concentration- and time-dependent manner, whereas the secretion of IL-6 and tumor necrosis factor-α was not detectable. The increased production of IL-8 and expression of IL-8 mRNA were significantly inhibited by rebamipide (100–1000 μM) in a concentration-dependent manner. Formyl-methionyl-leucyl-phenylalanine and CM were significantly inhibited by rebamipide (100–1000 μM) in a concentration-dependent manner. Formyl-methionyl-leucyl-phenylalanine (1 nM), as well as conditioned medium (CM) that was produced from *H. pylori*-infected Kato III cells, caused an increase in surface expression of CD11b on human neutrophils and an increase in neutrophil adhesion to the human umbilical vein endothelial cells. Rebamipide also suppressed the adherence of neutrophils to endothelial cells as well as the expression of CD11b on neutrophils induced by formyl-methionyl-leucyl-phenylalanine and CM. Furthermore, CM-induced neutrophil adhesion to the endothelial cells was significantly inhibited by IL-8-neutralizing antibody, suggesting that IL-8 is implicated in the CM-induced neutrophil adhesion to the cultured human umbilical vein endothelial cells. It is concluded that rebamipide exerts its preventive effect against *H. pylori*-evoked gastric mucosal cell inflammation by inhibition of the neutrophil adherence to the endothelial cells as well as by suppressing the surface expression of CD11b on neutrophils and the production of proinflammatory cytokine such as IL-8 from gastric epithelial cells.

Several studies have suggested that neutrophils are the chief provocative cells in the development of inflammation and injury in a variety of tissues, including the gastric mucosa (Ninemann, 1988). Despite increasing evidence of a major role for neutrophils in the tissue injury, the mechanism or mechanisms underlying activation and recruitment of these cells into the tissue are not clear. Recent studies have demonstrated that cultured human gastric epithelial cells are capable of expressing and releasing several proinflammatory cytokines, including IL-8, that directly or indirectly influence the activity of neutrophils.

It is known that *Helicobacter pylori* bacteria are closely associated with gastric and duodenal ulcer diseases (Graham, 1991; Cover and Blaser, 1992; Megraud and Lamouliatte, 1992). Yoshida et al. (1993) and Evans et al. (1995) have reported that the products of *H. pylori* elicit gastrointestinal inflammation by promoting neutrophil adhesion to endothelial cells via CD11b/CD18-dependent interactions with ICAM-1 on endothelium. Furthermore, *H. pylori* intimately adheres to the surface of gastric epithelium (Hessey et al., 1990) and stimulates production of chemoattractants from the epithelial cells (Crawtree et al., 1994; Crowe et al., 1995). In line with these facts, IL-8 activity was also demonstrated in relation with histological severity in *H. pylori*-associated antral gastritis (Ando et al., 1996; Crabtree, 1996).

Rebamipide (2-(4-chlorobenzylamino)-3-[2-(1H)-quinolinon-4-yl]propionic acid) has been reported to exert a preventive effect on the drug-induced gastric ulcer formation by inhibiting neutrophil activation (Ogino et al., 1992) and lipid peroxidation (Yoshikawa et al., 1993). Recently, we have reported that rebamipide prevents gastric lesions induced by ischemia-reperfusion via inhibition of the production of reactive oxygen species from activated neutrophils (Kim and Hong, 1995). More recently, we found that the inhibitory effect of rebamipide on the neutrophil-mediated gastric mucosal cell injury was ascribed to the alterations in the neutrophil membranes, that is, a decrease in the number of binding sites for fMLP to its receptors (Kim and Hong, 1997).

**ABBREVIATIONS:** IL, interleukin; fMLP, formyl-methionyl-leucyl-phenylalanine; HUVEC, human umbilical vein endothelial cell; CM, conditioned medium; RT, reverse transcription; PCR, polymerase chain reaction; TNF, tumor necrosis factor; PBS, phosphate-buffered saline.
However, the mechanism or mechanisms by which rebamipide exerts its cytoprotective effect against gastric cell damage are not yet fully determined.

In the present study, our goal was to investigate the underlying mechanism or mechanisms of the cytoprotective action of rebamipide against *H. pylori*-infected gastric mucosal cell damage. Thus, we measured the effects of rebamipide on (1) the production of IL-8 and expression of IL-8 mRNA in the Kato III cells in response to *H. pylori*, (2) the surface expression of CD11b on human neutrophils activated by either fMLP and CM, and (3) the fMLP- and CM-stimulated neutrophil adherence to the HUVECs in comparison with the effects of IL-8-neutralizing antibody.

**Materials and Methods**

**Culture of Gastric Epithelial Cell Line.** Kato III cells (gastric carcinoma; ATCC HTB 103) as human cell line was purchased from the American Type Culture Collection (Rockville, MD). Cells were grown in a RPMI 1640 medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were seeded onto 24-well tissue culture plates at a density of 2 × 10⁵ cells per well and cultured for 3 days. Before *H. pylori* stimulation, each well was washed three times with 1 ml of fresh antibiotic-free culture medium. The number of epithelial cells seeded and days of growth required to achieve this final number of cells were kept constant so that any effect of cell growth would contribute minimally to any changes in cytokine production.

**Culture of Endothelial Cells.** HUVECs (ATCC HB-11608) were cultured in Medium 199 (GIBCO, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum, heparin sodium (90 μg/ml), antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin), and endothelial cell growth supplement (150 μg/ml; Sigma Chemical, St. Louis, MO). Cells were grown to confluence at 37°C on 0.5% gelatin-coated 24-well cell culture plates and used for experiments at no greater than passage 8.

**Culture of *H. pylori***. *H. pylori* (ATCC 43526) was inoculated on a chocolate agar plate and incubated for 72 h in a microaerobic and humidified atmosphere at 37°C. Whole *H. pylori* was harvested from a culture plate with sterile cotton swabs, resuspended in the antibiotic-free RPMI 1640 medium (10⁵ bacteria/ml), and added to the cultured Kato III cell medium at various bacteria-to-Kato III cell ratios (1:1, 10:1, and 100:1). For collection of CM, Kato III cells were cocultured with *H. pylori* for 24 h at a bacteria-to-Kato III cell ratio of 100:1. To remove *H. pylori* from the CM, CM obtained was centrifuged at 1400g for 20 min, and the supernatant was passed through a 0.2-μm syringe-adapter filter.

**Cytokine Assay.** The levels of IL-6, IL-8, and TNF-α in the culture supernatants were assayed with the ELISA kits (R and D Systems, Minneapolis, MN). The supernatant (50 μl) and buffer standard solution were dispersed into microtiter wells coated with the polyclonal antibody specific for IL-6, IL-8, and TNF-α, respectively. After a 2-h incubation period at room temperature, the wells were washed, and 100 μl of the peroxidase-conjugated secondary antibody was added. After a 2-h incubation at room temperature, the wells were washed, and 100 μl of peroxidase substrate was added to each well, after which the absorbance at 450 nm was measured.

**RT-PCR Amplification for IL-8 mRNA.** IL-8 mRNA gene expression was assayed using RT-PCR standardized by coamplifying IL-8 with the housekeeping gene GAPDH, which served as an internal control because it must control for variations due to sample preparation. Cellular RNA was extracted from the Kato III cells infected in vitro with *H. pylori*. RNA (2 μg) was reverse-transcribed into cDNA at 42°C for 1 h. Then, the cDNA (25 μl) was amplified with the commercially available PCR primers (Clontech Laboratories, Palo Alto, CA) for IL-8 or GAPDH in a 50-μl reaction mixture, which contained Taq polymerase for 30 cycles of 45 s at 94°C, 45 s at 60°C, and 2 min at 72°C in a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, CO). As a negative control for PCR amplification, the cDNA sample was omitted from the reaction mixture. The amplified products were subjected to electrophoresis in a 1.8% agarose gel containing ethidium bromide and were visualized by UV transillumination. Results were normalized based on the levels of GAPDH expression.

**Immunofluorescence Flow Cytometry.** Surface expression of CD11b on the neutrophils was determined by immunofluorescence flow cytometry. Briefly, 10⁶ neutrophils purified from human peripheral blood were incubated with either fMLP or CM for 30 min at 37°C. Rebamipide was added 15 min before stimulation with fMLP or CM. Subsequently, the neutrophils were washed with PBS, treated with 10 μl of the phycoerythrin-conjugated monoclonal antibody against CD11b (clone D12; Becton Dickinson), and then incubated for 60 min in the dark at 4°C. Thereafter, the cells were washed with PBS containing 0.1% azide, fixed with 0.5 ml of 1% paraformaldehyde, and then analyzed using FACS Vantage (Becton Dickinson). The data were expressed as percentages of total fluorescent intensity (TFI) of the surface antigen expression. TFI was calculated as TFI = % positive events × mean channel of fluorescence.

**Isolation of Neutrophils.** Human neutrophils were isolated from 0.2% EDTA-anticoagulated whole blood collected by venipuncture from normal donors. Neutrophils were obtained using standard dextran sedimentation and gradient separation on Histopaque 1077 (Sigma Chemical). This procedure yields a polymorphonuclear leukocyte population that is 95% to 98% viable (trypan blue exclusion) and 98% pure (acetic acid-crystal violet staining).

**Neutrophil Adhesion Assay.** Isolated neutrophils were suspended in PBS and radiolabeled by incubation of the neutrophils (2 × 10⁶ cells/ml) with 30 μCi of Na¹¹⁵CrO⁴/ml at 37°C for 60 min. The cells were washed twice with cold PBS at 250g for 8 min to remove unincorporated radioactivity and then resuspended in plasma-free PBS. Radiolabeled neutrophils were added to HUVEC monolayers at a neutrophil-to-endothelial cell ratio of 10:1 with or without stimulants (1 mM fMLP or CM). After incubation at 37°C for 30 min in the presence or absence of rebamipide (100–1000 μM) or IL-8-neutralizing antibody (1, 3, and 10 μg/ml), the supernatant was removed; then, the endothelial monolayers were washed, and the remaining cells were lysed. The ¹¹⁵Cr activities of the supernatant, wash fluid, and lysate were assessed in a gamma counter (Wallac 1470 Wizard).

The percentage of added neutrophils that adhered to the HUVEC monolayers was determined as follows:

Neutrophil adherence (%) =

\[ \text{Lysate (cpm)} \times 100 \]

\[ \text{Supernatant (cpm) + Wash (cpm) + Lysate (cpm)} \]

**Drugs.** Rebamipide (Otsuka Pharmaceutical, Tokushima, Japan) was dissolved in 10 mM NaOH solution at a concentration of 10 mM. fMLP and anti-human IgG (Fab specific) were purchased from Sigma Chemical. Anti-human IL-8 (purified mouse monoclonal IgG1), was obtained from R and D Systems.

**Statistics.** All data are expressed as mean ± S.E.M. Statistical differences between groups were determined by Student’s *t* test. Differences were significant when *P* < .05.

**Results**

**Cytokine Production by *H. pylori*-Stimulated Kato III Cells.** Analysis of the cytokine production in the culture medium demonstrated that Kato III cells constitutively released 9.2 ± 2.2 ng/10⁶ cells of IL-8, whereas IL-6 and TNF-α were not detectable. After exposure to *H. pylori*, the secretion of IL-8, but not IL-6 and TNF-α, from Kato III cells was
markedly increased with increased number of bacteria. In the preliminary experiment, IL-8 was not detectable in the culture media of H. pylori alone (Fig. 1). Secretion of IL-8 from Kato III cells largely increased with increased number of bacteria, up to 400%, at ratios of 10 and 100 H. pylori:1 Kato III cell (P < .05 and P < .01, respectively). Therefore, in this experiment, a ratio of 100 bacteria:1 Kato III cell was routinely applied.

Fig. 2 shows time-dependent increases in IL-8 secretion from Kato III cells (10⁶ cells) when cocultured with H. pylori (10⁸/well). IL-8 production was not detectable in the culture supernatant within 1 h of the addition of H. pylori. The active secretion of IL-8 occurred from 3 h and reached a plateau (10⁸/well) Kato III cells, respectively. In a separate experiment, the increased IL-8 production (39.6 ± 3.0 ng/10⁶ Kato III cells) induced by H. pylori at a ratio of 100 bacteria:1 Kato III cell was significantly attenuated by 300 and 1000 μM rebamipide, respectively (Fig. 3).

PCR Analysis of IL-8 mRNA Expression in Kato III Cells. The potential of H. pylori to stimulate IL-8 synthesis from Kato III cells was studied by RT-PCR analysis. Figure 4 shows a representative RT-PCR analysis of IL-8 and GAPDH mRNA. Consistent with the secretion of IL-8, which was exhibited in the culture media, analysis of the mRNA expression demonstrated that Kato III cells had a very low level of IL-8 mRNA expression in H. pylori-free state (Fig. 4a). The IL-8/GAPDH mRNA ratios markedly increased in the H. pylori-infected cells in a concentration-dependent manner from 10⁶ to 10⁸ H. pylori/10⁶ Kato III cells (Fig. 4, a and b). The level of IL-8 mRNA also increased with increased infection time from 3 to 24 h (data not shown). The increase in IL-8 mRNA expression in the H. pylori-infected Kato III cells was significantly suppressed by rebamipide (300 and 1000 μM; P < .05, respectively) in a concentration-dependent manner (Fig. 5, a and b).

Expression of CD11b on Neutrophils. Under stimulation with fMLP (1 nM), CM, and fMLP plus CM, neutrophils showed a large increase in the surface expression of CD11b on their membranes. The ability of fMLP plus CM to induce CD11b expression was comparatively larger than that of fMLP or CM alone (Fig. 6, inset). The increased expression of CD11b stimulated by either fMLP or CM was significantly suppressed by pretreatment with rebamipide (300 and 1000 μM) in a concentration-dependent manner. However, the basal expression of CD11b on neutrophils, unless stimulated, was not affected by rebamipide (Fig. 6).

Neutrophil Adherence to Endothelial Cells. The percentage of total neutrophils adhering to the cultured HUVECs was significantly increased in the presence of fMLP (1 nM) and CM. Increased neutrophil adhesion stimulated by either fMLP or CM was significantly suppressed by rebamipide (300 and 1000 μM) in a concentration-dependent manner (Fig. 7). The viability of neutrophils and HUVECs was not affected by the addition of 1000 μM rebamipide to the medium.

Effect of IL-8-Neutralizing Antibody on Neutrophil Adhesion. As shown in Fig. 8, CM significantly enhanced the percentage of neutrophil adherence to the cultured HUVECs, which was concentration-dependently suppressed by IL-8-neutralizing antibody as it was by rebamipide, whereas anti-human IgG exerted little effect, thereby suggesting that IL-8 secretion from gastric epithelial cells may
be implicated in the CM-induced neutrophil adhesion to the endothelium.

**Discussion**

A number of experiments indicate that infection with *H. pylori* is characterized by elevation of mucosal inflammatory cytokines such as IL-6, IL-8, and TNF-α (Crabtree et al., 1994; Gionchetti et al., 1994; Noach et al., 1994) in association with neutrophil-mediated tissue injury (Wallace, 1990; Blaser, 1992). In the present study, rebamipide significantly inhibited (1) the *H. pylori*-evoked production of IL-8 and expression of IL-8 mRNA in the Kato III cells, (2) the surface expression of CD11b on neutrophils stimulated by either fMLP or CM, and (3) the fMLP- or CM-stimulated neutrophil adherence to the HUVECs. It has been reported that rebamipide has an oxygen radical-scavenging effects (Yoshikawa et al., 1993), and it prevents gastric ulcers induced by ethanol and diethyldithiocarbamate, which appear to be the conditions that increase oxidative stress in the organs (Takeuchi et al., 1996). Clinically, rebamipide has been tried with some efficacy against the patients with gastritis and/or gastric ulcer infected by *H. pylori* (Davies et al., 1994). IL-8 is secreted by several cell types, including monocytes, endothelial cells, and gastric epithelial cells (Baggiolini et al., 1989; Yasumoto et al., 1992), and plays a role for potent inflammatory mediator by activating neutrophils (Huber et al., 1991). Our present study showed that IL-8 production was significantly increased in the culture medium of *H. pylori*-infected Kato III cells in a *H. pylori* density-dependent manner, but those of IL-6 and TNF-α were not detectable. These results were consistent with the report of Huang et al. (1995). They conducted the RT-PCR analysis of the synthesis of IL-6, IL-8, and TNF-α in Kato III cells in response to *H. pylori* (CCUG 17874), and they found no expression of mRNA for IL-6 and TNF-α, whereas mRNA for IL-8 was highly expressed in response to *H. pylori*. The results suggest that IL-8 generated by Kato III cells may be closely related with the gastritis caused by *H. pylori* infection.

In further study, the increases in IL-8 production and expression of IL-8 mRNA in Kato III cells were significantly inhibited by rebamipide, suggesting that rebamipide may suppress the synthesis of IL-8 in the *H. pylori*-infected gastric mucosal cells. These results provide the evidence that rebamipide can protect gastric mucosal cells from damage induced by *H. pylori* infection through inhibition of production of IL-8 from gastric mucosal cells.

On the other hand, the CD11b/CD18 glycoprotein complex expressed on neutrophils is known as one of the important mediators of neutrophil adhesion to endothelial cells in inflammation (House and Lipowsky, 1987; Diamond et al., 1990; Yoshida et al., 1993). CD11b/CD18 glycoprotein is stored within specialized granules of resting neutrophils and, when activated, is translocated to the cell surface by granule fusion (Bainton et al., 1987). Neutrophil activation also triggers functional activation of preexisting cell surface CD11b/CD18, presumably through conformational or topological alterations (Buyon et al., 1988). In the present study, we used fMLP and CM for activation of neutrophils. The latter was produced from *H. pylori*-infected Kato III cells as a stimu-
CM as well as fMLP (1 nM) caused a large increase in the surface expression of CD11b on the neutrophil membranes, and the increased expressions of CD11b were significantly suppressed by rebamipide (300 and 1000 µM) in a concentration-dependent manner. These results suggest that rebamipide possesses an ability to inhibit the neutrophil adhesion to endothelial cells by inhibiting the surface expression of CD11b on neutrophils, thereby suppressing the acute inflammation evoked by activated neutrophils.

Our results further demonstrated that not only fMLP but also CM largely increased the neutrophil adhesion to HUVECs and that these adhesive interactions were significantly suppressed by rebamipide. At present time, it is not clear whether fMLP itself or an fMLP-related substance in CM is implicated as a chemotactic factor in H. pylori-induced inflammation. In a study directed to explain the mechanism by which H. pylori recruits inflammatory cells, Mooney et al. (1991) have shown that H. pylori synthesizes and secretes fMLP as a chemotactic substance. Reportedly, stimulation of neutrophils with chemotactic agents leads to increased expression of CD11b/CD18 on neutrophils (House and Lipowsky, 1987; Diamond et al., 1990). Yoshida et al. (1993) also postulated that the hyperadhesive response induced by the H. pylori extract was dependent on CD11a/CD18 on neutrophils. CM as well as fMLP (1 nM) caused a large increase in the surface expression of CD11b on the neutrophil membranes, and the increased expressions of CD11b were significantly suppressed by rebamipide (300 and 1000 µM) in a concentration-dependent manner. These results suggest that rebamipide possesses an ability to inhibit the neutrophil adhesion to endothelial cells by inhibiting the surface expression of CD11b on neutrophils, thereby suppressing the acute inflammation evoked by activated neutrophils.

Our results further demonstrated that not only fMLP but also CM largely increased the neutrophil adhesion to HUVECs and that these adhesive interactions were significantly suppressed by rebamipide. At present time, it is not clear whether fMLP itself or an fMLP-related substance in CM is implicated as a chemotactic factor in H. pylori-induced inflammation. In a study directed to explain the mechanism by which H. pylori recruits inflammatory cells, Mooney et al. (1991) have shown that H. pylori synthesizes and secretes fMLP as a chemotactic substance. Reportedly, stimulation of neutrophils with chemotactic agents leads to increased expression of CD11b/CD18 on neutrophils (House and Lipowsky, 1987; Diamond et al., 1990). Yoshida et al. (1993) also postulated that the hyperadhesive response induced by the H. pylori extract was dependent on CD11a/CD18 on neutrophils.

**Fig. 6.** Effect of rebamipide on the CD11b expression stimulated by fMLP (a) and CM (b) on human neutrophils. Inset, expression of CD11b on neutrophil membranes on stimulation of fMLP (1 nM), CM, and fMLP plus CM in comparison with basal level. Basal represents the expression of CD11b in the absence of stimulation. Vehicle represents the results obtained in the absence of rebamipide. All values are expressed as mean ± S.E.M. from four or five experiments. *, P < .001 versus basal; **, P < .01 versus vehicle.

**Fig. 7.** Effect of rebamipide on the neutrophil adherence to HUVECs. The neutrophil adherence to HUVECs was stimulated by either (a) fMLP (1 nM) or (b) CM. Basal represents the neutrophil adherence in the absence of stimulation. Vehicle represents the results obtained in the absence of rebamipide. All values are expressed as mean ± S.E.M. from four experiments. *, P < .01 versus basal; **, P < .01 versus vehicle.

**Fig. 8.** Effect of IL-8-neutralizing antibody (1, 3, and 10 µg/ml) on the neutrophil adherence to HUVECs, which was stimulated by CM. Basal represents the neutrophil adherence in the absence of stimulation. Vehicle represents the results obtained in the absence of anti-IL-8-neutralizing antibody. Anti-human IgG was used for comparison. All values are expressed as mean ± S.E.M. from four experiments. *, P < .01 versus basal; **, P < .01 versus vehicle.
phils. We recently explored how rebamipide exerts its inhibitory effect on FMLP-stimulated neutrophil functions such as aggregation, release of myeloperoxidase, and superoxide anion production (Kim and Hong, 1995). These findings were ascribed to the decreased binding of [3H]FMLP to its receptors on neutrophils (Kim and Hong, 1997). Interestingly, CM-induced increase in neutrophil adhesion to the endothelial cells was significantly inhibited by IL-8-neutralizing antibody, as it was by rebamipide. This result strongly suggests that IL-8 secreted from gastric epithelial cells infected by H. pylori may be implicated in the signal transduction of the CM-induced neutrophil adhesion to the cultured HUVECs.

In summary, rebamipide exerts the preventive effects against H. pylori-infected gastric cell damage by inhibiting the production of epithelial cell-derived proinflammatory cytokines such as IL-8 and by suppressing the FMLP- or CM-induced surface expression of CD11b on neutrophils, thereby inhibiting adhesion and migration of neutrophils. Further investigation to identify the molecular mechanism or mechanisms involved in the suppression of IL-8 and CD11b expression by rebamipide may provide more valuable information for the therapeutic approaches to gastritis and gastric and duodenal ulcers in response to a variety of pathogens.

Acknowledgments

The authors thank Dr. Dai Hyun Yu for his critical review of the manuscript. The gift of rebamipide from Otsuka Pharmaceutical Co. Ltd. (Japan) is greatly acknowledged.

References


Send reprint requests to: Ki Whan Hong, M.D., Doctor of Philosophy Department of Pharmacology, College of Medicine, Pusan National University, Ami-Dong 1-Ga, Seo-Gu Pusan 620-729, Korea. E-mail: kwong@hyowon.pusan.ac.kr