Inhibitory effect of polaprezinc on the inflammatory response to Helicobacter pylori

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Helicobacter pylori-infected gastrointestinal mucosa is frequently infiltrated by polymorphonuclear leukocytes (PMN) and monocytes, and these invading cells have been implicated in gastrointestinal mucosal inflammation. To clarify the efficacy of polaprezinc, a chelate compound consisting of zinc and L-carnosine, against H pylori-induced inflammation including PMN infiltration, the in vitro effects of this drug on interleukin (IL)-8 production by an established gastric cancer cell line (MKN 45 cells) and on PMN-endothelial cell adhesive interactions was investigated. Polaprezinc and zinc sulphate inhibited IL-8 production by MKN 45 cells in response to stimulation with H pylori water extract (HPE) in a dose-dependent manner from $10^{-7}$ M to $10^{-5}$ M. In addition, the expression of CD11b and CD18 on PMN and PMN-dependent adhesion to endothelial cells elicited by HPE was inhibited by polaprezinc and zinc sulphate in a concentration-dependent manner. L-carnosine did not have any effects on IL-8 production or PMN-endothelial cell interactions. These results suggest that polaprezinc, mainly the zinc component, may inhibit H pylori-induced PMN-mediated gastric inflammation by attenuating CD11b/CD18 expression on PMN and IL-8 production from gastric epithelial cells.

Key Words: CD11b/CD18; Gastric epithelial cells; Helicobacter pylori; Interleukin-8; Polaprezinc; Polymorphonuclear leukocytes

L'effet inhibiteur du polaprézinc sur la réaction inflammatoire à l'Helicobacter pylori

RÉSUMÉ : La muqueuse gastro-intestinale infectée par l'Helicobacter pylori est souvent infiltrée par des leucocytes polymorphonucléaires (LPM) et par des monocytes, et ces cellules envahissantes participent à l'inflammation de la muqueuse intestinale. Pour clarifier l'efficacité du polaprézinc, on a examiné un chélate, composé de zinc et de L-camosine, contre l'inflammation à l'Helicobacter pylori, y compris l'infiltration par les LPM ainsi que les effets in vitro de ce médicament sur la production d'interleukine (IL)-8 par une lignée cellulaire cancéreuse (cellules MKN 45) et sur les interactions des adhésions cellulaires endothéliales aux LPM. Le polaprézinc et le sulfate de zinc anhydre ont inhibé la production d'IL-8 par les cellules MKN 45 après stimulation par un extrait aqueux de H pylori (EHP), de manière proportionnelle à la dose administrée de $10^{-7}$ M à $10^{-5}$ M. De plus, l'expression du CD11b et du CD18 sur les LPM et l'adhésion proportionnelle aux LPM sur les cellules endothéliales sollicitées par l'EHP étaient inhibées par le polaprézinc et le sulfate de zinc anhydre selon la concentration. Le L-camosine n'avait aucun effet sur la production d'IL-8 ou sur les interactions cellulaires endothéliales des LPM. Ces résultats laissent supposer que le polaprézinc, et surtout la particule de zinc, inhiberait peut-être l'inflammation gastrique assistée par les LPM secondaires au H pylori en atténuant l'expression du CD11b et du CD18 sur les LPM et la production d'IL-8 par les cellules épithéliales gastriques.
A number of studies have indicated that Helicobacter pylori isolated from human gastric mucosa is the cause of gastrointestinal mucosal lesions such as gastroduodenal ulcers and chronic active gastritis (1,2). Infiltration of polymorphonuclear leukocytes (PMN) into the mucosa is known to be a characteristic finding in H. pylori-induced chronic active gastritis (3,4). Eradication of H. pylori has been reported to significantly reduce PMN infiltration (5), suggesting a correlation between H. pylori infection and PMN invasion. This organism is presumed to cause PMN infiltration through some mediators, because it normally resides chiefly in the mucosal layer covering the mucosal epithelium.

Recently, it was reported that H. pylori organism or H. pylori water extract (HPE) enhanced interleukin (IL)-8 production by gastric mucosal epithelial cells (6-8), and that HPE promoted PMN attachment to vascular endothelial cells (9,10). This hyperadhesiveness has been shown to involve the expression of adhesion molecules (CD11a/CD18 and CD11b/CD18) on PMN cell membranes, which can be elicited by HPE (9). Based on these findings, H. pylori infecting the gastric mucosa appears to cause PMN to migrate and infiltrate via the promotion of IL-8 production by epithelial cells and through the induction of adhesion molecule expression on PMN.

Polaprezinc, a chelate compound consisting of zinc and L-carnosine, is a unique antiulcer drug that adheres to and resides chiefly in the mucous layer covering the mucosal epithelium. Several reports, including studies from our group, have shown that polaprezinc protects against experimental gastric mucosal injury in animal models through inhibiting lipid peroxidation and neutrophil infiltration (11-14); however, mechanisms of anti-inflammatory effects of polaprezinc are not clear.

We investigated the effects of polaprezinc, zinc sulphate and L-carnosine on IL-8 production by human gastric epithelial cells, and on the expression of CD11b/CD18 on PMN and PMN adherence to endothelial cells, which are induced by HPE.

**MATERIALS AND METHODS**

**Test drugs**
Polaprezinc (Zeria Pharmaceuticals, Japan), L-carnosine (Zeria Pharmaceuticals, Japan) and zinc sulphate (Wako Pure Chemical Industry, Japan) were used. Polaprezinc was dissolved in two equivalents of hydrochloride and then diluted with Hank’s balanced salt solution (HBSS) to the designated concentrations. L-carnosine and zinc sulphate were also dissolved in the same method. All other reagents used were of special grade.

**H. pylori water extract**
HPE was prepared, as previously described (9), from standard strain NCTC 11637. Briefly, the organism was grown on blood agar plates and harvested with sterile cotton swabs into distilled water, using 1.0 mL per plate (10⁶ to 10⁹ bacteria). The cell suspension was kept at room temperature for 20 min before centrifugation at 25,000 g for 15 min. The resultant supernatant was the initial water extract; no preservatives were added, and the extract was stored at -70°C until needed. Before use, the water extract was thawed at room temperature and centrifuged at 55,000 g for 20 min. To remove much of the high molecular weight material that consisted mainly of membrane vesicles and whole flagellae, the supernatant was clarified by passage through a 0.2 µm syringe-adapter filter.

**Preparation of human PMN**
Heparinized peripheral blood collected from healthy adults was subjected to dextran sedimentation and density gradient centrifugation using Ficoll-Paque (Pharmacia Biotech AB, Sweden) to isolate PMN, which were suspended in HBSS. This procedure yields a PMN population that is 95% to 98% viable (trypan blue exclusion) and 98% pure (acetic acid-crystal violet staining).

**Endothelial cells**
Human umbilical vein endothelial cells (HUVEC) were harvested from umbilical cords by collagenase treatment, as previously described (15). The cells were plated in Medium 199 (Gibco, USA) supplemented with 10% heat-inactivated fetal calf serum (Hyclone Laboratories Inc, USA), thymidine (2.4 mg/L, Sigma Chemical, USA), glutamine (230 mg/mL, Gibco, USA), heparin sodium (10 IU/mL, Sigma Chemical, USA), antibiotics (100 IU/mL penicillin, 100 µg/mL streptomycin and 0.25 µg/mL amphotericin B, Gibco, USA) and endothelial cell growth factor (80 µg/mL, Biomedical Technologies Inc, USA). The cell cultures were incubated at 37°C in a humidified atmosphere with 5% carbon dioxide and expanded by brief trypsinization (0.25% trypsin in phosphate-buffered saline containing 0.02% ethylenediamine tetra-acetic acid). HUVEC of the primary through the third passage were seeded into 0.1% gelatin and 25 µg/mL fibronectin-coated, 96-well tissue culture plates (Gibco, USA) and used when confluent.

**Cell viability**
To investigate the effect of polaprezinc, zinc sulphate or L-carnosine on the viability of PMN or MKN 45 cells, the cells were incubated with each compound at a final concentration of 10⁻⁵ M for 30 min and 6 h, respectively. Cell viability was assessed by trypan blue dye exclusion.

**IL-8 production by MKN 45 cells**
MKN 45 cells (8), a human gastric cancer cell line, were cultured in RPMI 1640 medium (Gibco, USA) containing 10% fetal calf serum (JRH Biosciences, USA) and antibiotics (100 IU/mL penicillin, 100 µg/mL streptomycin and 0.25 µg/mL amphotericin B, Gibco, USA) and then incubated at 37°C in a humidified atmosphere with 5% carbon dioxide.

MKN 45 cells were seeded into 48 plates (Sumitomo Bakelite Co Ltd, Japan) at 2×10⁵ cells per plate and incubated under the conditions described above. When the cells became confluent approximately 72 h after seeding, HPE (final concentration 2%) was added. The plates were incu-
reaction mixture consisted of 5 µL of 10 mM sodium azide (Dako Co, USA). The wells were washed with phosphate buffered solution (PBS), and a fluorescein isothiocyanate-conjugated goat F(ab')2 fragment specific for mouse immunoglobulin G (Cappel, USA) was added at a final concentration of 10 µg/mL. After a 15 min incubation at 4°C, PMN were washed with PBS, and monoclonal antibodies binding to the cells were analyzed in an EPICS Profile flow cytometer (Coulter Corp, USA). To determine the effect of polaprezinc, zinc sulphate or L-carnosine on the HPE-induced adhesion of PMN, each compound was simultaneously added to the plate with HPE. HBSS with the same concentration of hydrochloride as that of tested drugs was used as the vehicle of the drug.

Expression of adhesion molecule on PMN
Surface expression of CD18, the β2 subunit of CD11/CD18 glycoprotein, on PMN was determined by immunofluorescence flow cytometry, as previously described (10). Briefly, the reaction mixture consisted of 5×10^5 PMN, HPE (final concentration 2%), monoclonal antibody (final concentration 2 mg/mL) to CD18 (L130, Becton Dickinson, USA) or CD11b (D12, Becton Dickinson, USA) in a total volume of 500 µL of HBSS. After a 30 min incubation at 37°C, PMN were washed with phosphate buffered solution (PBS), and a fluorescein isothiocyanate-conjugated goat F(ab')2 fragment specific for mouse immunoglobulin G (Cappel, USA) was added at a final concentration of 10 µg/mL. After a 15 min incubation at 4°C, PMN were washed with PBS, and monoclonal antibodies binding to the cells were analyzed in an EPICS Profile flow cytometer (Coulter Corp, USA). To determine the effect of polaprezinc, zinc sulphate or L-carnosine on CD11b or CD18 expression on PMN in response to stimulation with HPE, each compound was added simultaneously with the primary antibodies just before adding HPE. HBSS with the same concentration of hydrochloride as that of tested drugs was used as the vehicle of the drug.

PMN adhesion assays
PMN adhesion to endothelial cells grown in 96-well culture plates was measured by enzyme immunoassay (16,17). PMN suspension (2×10^5 PMN) was added to each well along with HPE (final concentration 2%). After a 30 min incubation at 37°C in a carbon dioxide incubator, the cells were washed three times to remove nonadherent PMN, and adherent PMN were fixed with 1% paraformaldehyde in PBS for 15 min and washed. Anti-CD11a monoclonal antibody was added to wells and the resulting mixture was incubated for 30 min. After washing, the wells were incubated for 15 min with biotinylated antimouse immunoglobulin in PBS containing carrier protein and 15 mM sodium azide (Dako Co, USA). The wells were washed, horseradish peroxidase-conjugated streptavidin was added for 15 min, and the wells were washed again. Subsequently, 200 µL of 0.4 mg/mL orthophenylenediamine dihydrochloride (Sigma) in citrate buffer (pH 5) with 0.012% hydrogen peroxide was added. The reaction was stopped by the addition of 50 µL of 1.5 M sulphuric acid. The absorbance was then measured at 492 nm using a microplate reader (Tosoh Corp, Japan) to quantify the amount of antibody bound to adherent PMN. To determine the effect of polaprezinc, zinc sulphate or L-carnosine on the HPE-induced adhesion of PMN to endothelial cells, each compound and PMN were simultaneously added to endothelial monolayers just before adding HPE. HBSS with the same concentration of hydrochloride as that of tested drugs was used as the vehicle of the drug.

Statistical analysis
Results are expressed as the mean ± SE. Data were compared using analysis of variance, followed by Scheffe's test, as appropriate. Differences were considered significant at P<0.05.

RESULTS
Cell viability
The incubation of PMN or MKN 45 cells with HBSS (control), polaprezinc, zinc sulphate or L-carnosine resulted in a greater than 97% viability of cells, indicating that not all compounds affected cell viability (Table 1).

IL-8 production by MKN 45 cells
The effect of polaprezinc on IL-8 production by MKN 45 human gastric cancer cells after exposure to HPE was assessed. As shown in Figure 1, incubation of MKN 45 cells with HPE caused a marked increase of IL-8 in the culture supernatant. On the other hand, IL-8 production by MKN 45 cells was inhibited significantly by treatment with polaprezinc (1×10^-6 M and 1×10^-5 M) or zinc sulphate (1×10^-6 M and 1×10^-5 M), but not L-carnosine.

A dhesion molecule expression on PMN
The effects of polaprezinc and its components on the expression of CD11b and CD18 on PMN in response to stimulation with HPE were evaluated by using immunofluorescence flow cytometry. As indicated in Figures 2 and 3, the expression of CD11b and CD18 on PMN was increased significantly by exposure to HPE. Polaprezinc (1×10^-7 M to 1×10^-5 M) had a concentration-dependent inhibitory effect on the expression of CD11b and CD18. At the same concentration, L-carnosine had no inhibitory effect, but zinc sulphate had a suppressive effect similar to that of polaprezinc.

### TABLE 1

<table>
<thead>
<tr>
<th>Effect of polaprezinc and its compound on cell viability</th>
<th>Control</th>
<th>Polaprezinc (10^-5 M)</th>
<th>Zinc sulphate (10^-5 M)</th>
<th>L-carnosine (10^-5 M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polymorphonuclear leukocytes</td>
<td>99.0</td>
<td>97.2</td>
<td>97.1</td>
<td>98.8</td>
</tr>
<tr>
<td>MKN-45</td>
<td>97.5</td>
<td>98.8</td>
<td>97.9</td>
<td>99.8</td>
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</tbody>
</table>
PMN adhesion to HUVEC

Figure 4 shows the effect of polaprezinc and its components on PMN adherence to HUVEC caused by HPE. HPE increased PMN-endothelial cell adhesive interactions, and polaprezinc and zinc sulphate reduced the adherence of HPE-stimulated PMN to HUVEC in a dose-dependent manner, but L-carnosine had no effect on PMN adhesion.

DISCUSSION

Infection of gastric mucosa with H. pylori has been shown to be associated closely with PMN infiltration, which is thought to be a cause of progression to gastric mucosal injury (3,4). The process by which H. pylori infection promotes PMN infiltration has been reported to involve IL-8 production from gastric epithelial cells by the organism (6-8) and surface expression of adhesion molecules on both circulating PMN and vascular endothelial cells (9). In the present study, we investigated the effect of polaprezinc on the inflammatory response to HPE in vitro. It has been reported that polaprezinc exhibits antiulcer effects by acting mainly on mucosal lesions (18). Polaprezinc shows a long-term adhesive and permeable action on the gastric mucosa in acetic acid ulcer rats at the concentration of $10^{-7}$ M to $10^{-5}$ M, and it has a comparable high affinity at the ulcerous site. It is probable that polaprezinc ($10^{-7}$ M to $10^{-5}$ M) has a direct effect on PMN and gastric epithelial cells and endothelial cells around ulcers in the gastric mucosa. Therefore, we used polaprezinc and its components at the concentration of $10^{-7}$ M to $10^{-5}$ M in the present in vitro study.

IL-8 is known as a chemotactic or activating factor for PMN, and it is reportedly produced by stimulation of viable H. pylori or HPE into cultured gastric mucosal cells (7,8). In the present study, IL-8 production was detected after the stimulation of MKN 45 cells with HPE. This IL-8 production was inhibited significantly by the addition of polaprezinc, suggesting that polaprezinc may prevent PMN...
infiltration into the gastric mucosa by suppressing IL-8 production in response to H. pylori infection.

The expression of adhesion molecules on PMN is important in the process of PMN infiltration into tissues. The attachment of PMN to vascular endothelial cells in the early phase of inflammation requires interactions between adhesion molecules on PMN and endothelial cells (19). We previously reported that augmented expression of a CD11b/CD18 glycoprotein complex on PMN is responsible for PMN adhesion caused by stimulation with HPE (9). In the present study, polaprezinc had a concentration-dependent inhibitory effect on both the expression of CD11b/CD18 and PMN adhesion induced by HPE. Thus, polaprezinc may inhibit PMN invasion in response to chemotactic factors derived from H pylori by suppressing expression of adhesion molecules on PMN. The CD11b/CD18 glycoprotein is stored within specialized granules of resting neutrophils and, when activated, is translocated to the cell surface by granule fusion (20). Neutrophil activation also triggers the functional activation of pre-existing cell surface CD11b/CD18, presumably through conformational or topological alterations (21). Although the exact mechanisms are unclear, it is possible that polaprezinc has a direct effect on either signal transduction pathways or translocation and conformational changes of the CD11b/CD18 complex.

We also evaluated the effects of zinc and L-carnosine, which are components of polaprezinc, on IL-8 production and PMN-dependent adhesive interactions. Although L-carnosine alone had no effect, zinc had an inhibitory effect similar to that of polaprezinc, indicating that the inhibitory effect of polaprezinc on IL-8 production, adhesion molecule expression and PMN adhesion might depend on zinc. These findings are consistent with the report by Connell et al (22) that zinc supplementation caused a marked attenuation in IL-8 expression by endothelial cells in response to tumour necrosis factor-alpha-mediated cell activation. Recently, Shimada et al (23) reported that polaprezinc inhibited cytokine-induced IL-8 production in gastric epithelial cells (MKN 28) by downregulating nuclear factor (NF)-kappa B activation. It has been proposed that IL-8 production from H. pylori-stimulated gastric epithelial cells also requires activation of protein kinase C (8) and tyrosine kinase (24) and, subsequently, activation of NF-kappa B and A P-1 (7). The exact mechanism through which polaprezinc suppresses IL-8 production and adhesion molecule expression induced by HPE has not been established, and further research on this topic is required.

Considering that polaprezinc suppressed IL-8 production by MKN 45 cells and the expression of adhesion molecules on PMN induced by HPE, this drug may be effective against gastric mucosal disease by preventing the PMN-mediated inflammatory response to H. pylori infection. Hence, polaprezinc has the potential to be very useful for the treatment of gastric mucosal disease associated with H pylori.

REFERENCES
