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Omeprazole promotes gastric epithelial cell migration

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Abstract

Proton pump inhibitors (PPIs) are effective at preventing non-steroidal anti-inflammatory drug (NSAID)-induced gastric ulcers. They are also superior to histamine H_2-receptor antagonists and misoprostol in treating NSAID-induced gastric ulcer healing. This study explored whether omeprazole, a PPI, can modulate ulcer healing through epithelial cell proliferation and/or cell migration using a rat normal gastric epithelial cell line (RGM-1). Flow cytometry was used to determine cell proliferation and an artificial wound model was used to measure cell migration. Western blot analysis was performed to evaluate the possible mechanisms of action. Omeprazole treatment (10^{-8}, 10^{-6} and 10^{-4} M) for 12 and 24 h did not promote cell proliferation. However, similar doses of the drug (10^{-6} and 10^{-4} M) incubated for 24–48 h significantly promoted the basal cell migration of gastric epithelial cells. Further, the higher concentration of omeprazole (10^{-4} M) reversed the inhibitory action of indometacin (10^{-5} M) on cell migration. Western blot results showed that omeprazole did not increase cyclooxygenase-2 expression and did not activate signal transduction pathways, including extracellular signal-regulated kinase (ERK1/ERK2), P38 mitogenic-activated protein kinase, and phosphatidyl inositol 3-kinase. The results suggest that omeprazole is beneficial in basal ulcer healing and it reversed the adverse action of indometacin on ulcer repair under acid-independent conditions. These actions are likely to be mediated through the promotion of gastric epithelial cell migration but not cell proliferation.

Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are known to cause gastric mucosal injury and ulcer formation in both human and animal studies (Wolfe et al 1999; Peskar 2002). The potential ulcerogenic mechanisms include: (i) inhibition of both cyclooxygenase (COX)-1 and COX-2 activity with decreased prostaglandin (PG) synthesis; (ii) an increase in the expression of intercellular adhesion molecule-1, with a consequent elevation of neutrophil infiltration and oxygen-derived free radical production; (iii) elevation of mucosal permeability; and (iv) induction of cytokine formation, for example leukotriene B_4 and tumour necrosis factor-α (Fiorucci et al 1993; Appleyard et al 1996; Okada et al 1998; Wolfe et al 1999; Wallace et al 2000). NSAIDs also delay ulcer healing by inhibiting COX activity and PG formation, which are important factors for mucosal repair in the stomach (Mizuno et al 1997; Peskar et al 2001; Peskar 2002; Luo et al 2003). PGs can stimulate cell migration (restitution) (Yoo et al 2002; Buchanan et al 2003), as well as cell proliferation and angiogenesis by activating the mitogenic and angiogenic transduction pathways (Pai et al 2002; Luo et al 2003, 2004).

Peptic ulcer formation is a dynamic state of imbalance between aggressive and protective factors. When the function of the protective and repairing factors is less than that of aggressive factors, mucosal injury worsens, ultimately resulting in the formation of ulcers. Following the acute gastric mucosal injury by aggressive factors, such as acid and NSAIDs, most of the mucosal components, including epithelial cells and micro-blood vessels, are destroyed within the focal lesion area. Healing of such lesions requires a reconstruction of the surface epithelium and glandular epithelial structures, restoration of the lamina propria and reconstruction of the microvascular network (Jones et al 1999). The early phase of mucosal repair occurs in the absence of cellular proliferation and is termed restitution or migration (Silen & Ito 1985; Yoo et al 2002). The late phase involves cell division and proliferation to fill in the

defect and restore mucosal architecture (Szabo et al 2000; Tarnawski et al 2001, 2005). All of these events are controlled by the cytokines, growth factors and some transcription factors (Szabo et al 2000; Tarnawski et al 2001; Tarnawski 2005).

Clinical studies show that proton pump inhibitors (PPIs) have been found to be superior to histamine H_2-receptor antagonists and misoprostol in the prevention and treatment of NSAID-associated ulcers (Ekstrom et al 1996; Cullen et al 1998; Hawkey et al 1998; Yeomans et al 1998; Agrawal et al 2000). Similarly, in animal studies, PPIs could reverse the deleterious actions of NSAIDs on gastric ulcer healing (Schmassmann 1998). It has been reported that PPIs have acid-independent mucosal protective actions (Blandizzi et al 1999; Tsuji et al 2002). PPIs also have anti-inflammatory actions via inhibitory effects on neutrophil–endothelial cell interactions and interfering with nuclear factor-κB activation (Yoshida et al 2000; Handa et al 2006). All these findings suggest that, in addition to the inhibitory action on gastric acid secretion, PPIs could have the ability to strengthen the gastric mucosal protection and promote mucosal repair through the activation of cell proliferation and/or migration of epithelial cells. This may account for the beneficial effect of PPIs as an adjuvant for NSAIDs in the treatment of inflammatory diseases.

In this in-vitro study, we investigated if omeprazole, a type of PPI, could stimulate gastric epithelial cell proliferation and/or cell migration in the presence of indometacin, one of the most potent NSAIDs used in clinical practice, in a rat normal gastric epithelial cell line (RGM-1).

Materials and Methods

Chemicals

All chemicals and reagents were purchased from Sigma (Sigma Chemical Company, St Louis, MO, USA) unless otherwise specified. Omeprazole was dissolved in dimethylsulfoxide (DMSO); indometacin was dissolved in ethanol with appropriate dilution (10^{-5} M) using the culture medium before experimentation.

Cell culture

Gastric epithelial cells obtained from the rat gastric mucosal epithelial cell line RGM-1 (RCB-0876 at Riken Cell Bank, Tsukuba, Japan) were grown in Dulbecco's modified Eagle's medium (DMEM)/F-12 medium (GibcoBRL, Grand Island, NY, USA) supplemented with 100 U mL^{-1} penicillin G, 100 μg mL^{-1} streptomycin and 20% fetal bovine serum (FBS) (GibcoBRL) in an incubator at 37°C and 5% carbon dioxide.

Cell number determination

RGM-1 cells were seeded in 10-cm dishes at approximately 10^5 cells mL^{-1} and were allowed to grow in DMEM/F-12 medium containing 20% FBS for 24 h. Thereafter, the growth of cells was arrested in the same medium with 2% FBS for a further 24 h to synchronize cell cycles. The cells were then treated for 12 and 24 h with 2% FBS DMEM/F-12 medium combined with omeprazole (10^{-8}, 10^{-6} and 10^{-4} M) or vehicle control (0.5% DMSO). The cells were detached from the wells after the experiments using 0.25% trypsin EDTA and were counted using a Burker haemocytometer (Marienfeld GmbH, Marienfeld, Germany).

Flow cytometry for DNA synthesis analysis

Cellular DNA replication was analysed by flow cytometry (Luo et al 2007). The two peaks of DNA content corresponding to G1- and G2/M-phase cells, respectively, and the intermediate amount of DNA corresponding to S-phase cells were counted. RGM-1 cells were seeded in 10-cm dishes at approximately 10^5 cells mL^{-1} and were allowed to grow in DMEM/F-12 medium containing 20% FBS for 24 h. Thereafter, the growth of cells was arrested in the same medium with 2% FBS for a further 24 h to synchronize cell cycles. The cells were then treated for 12 and 24 h with 2% FBS DMEM/F-12 medium combined with omeprazole (10^{-8}, 10^{-6} and 10^{-4} M) or vehicle control (0.5% DMSO). After treatment, the cells (approx. 2×10^6 in each dish) were trypsinized, pelleted, washed with phosphate buffered saline, re-pelleted and re-suspended with lysis buffer (0.5% Triton-X, 0.2 μg mL^{-1} $Na_2EDTA.2H_2O$, 1% bovine serum albumin) for 15 min. Subsequently, the cells were fixed in 80% cold methanol at –20°C overnight. The fixed cells were centrifuged, washed with phosphate buffered saline, pre-treated with RNase (5 Kunitz units mL^{-1}) at 37°C for 30 min, and then reacted with propidium iodine (50 μL mL^{-1}). The cells were analysed on a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA, USA). Data were analysed using ModFit and CellQuest software (Luo et al 2007).

Cell viability

Cell viability was measured using the 3-(4,5-dimethyl-thiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) reduction method (Luo et al 2005). After synchronization, cells were treated with 2% FBS DMEM/F-12 containing omeprazole (10^{-8}, 10^{-6} and 10^{-4} M), indometacin (10^{-5} M) or vehicle control (0.5% DMSO, 0.02% ethanol) for 24 h. The cells were then incubated with 2.5% MTT solution (5 mg mL^{-1}) for another 3 h at 37°C, then medium was aspirated, 0.04 M HCl–isopropanol was added and mixed thoroughly for 10 min. The colour change and optical density were determined by the MRX microplate reader (Dynex Technologies Inc., Chantilly, VA, USA) at 570 nm.

Cell migration

Cells were seeded in 24-well culture plates and cultured in DMEM/F-12 with 20% FBS until confluent. Then, monolayers of the cells were starved for 24 h in the medium containing 2% FBS. The cells were then pre-treated with mitomycin C (2 μg mL^{-1}) for 2 h before a wound was made to inhibit cell proliferation (Shin et al 2002). An artificial circular wound of cell-free area (2 mm^2) was made in the centre of the monolayer using a plastic blade (Shin et al 2002). The wounded monolayer was then cultured in the medium with 2% FBS in the presence of vehicle control (0.5% DMSO, 0.02% ethanol), omeprazole

(10^{-8}, 10^{-6} and 10^{-4} M), indometacin (10^{-5} M) or both omeprazole and indometacin. The size of the cell-free area was monitored from time 0 to 48 h using a digital image processor connected to a microscope (Nikon, Tokyo, Japan). The areas were calculated with an image-analysing program (Leica, Cambridge, UK).

Western blot analysis

After seeding, incubation and starvation similar to that carried out for flow cytometry, the cells were treated with 2% FBS DMEM/F-12 in the presence or absence of omeprazole (10^{-8}, 10^{-6} and 10^{-4} M) for 10 min, 30 min, and 1, 3, 6, and 12 h. The cells were then collected in radioimmunoprecipitation assay buffer for Western blot analysis. After sonication and centrifugation, the protein concentration was routinely measured using a protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis overlaid with acrylamide stacking gel, before being transferred to Hybond C nitrocellulose membranes (Amersham International plc, Amersham, UK).

The membranes were probed with antibodies against epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), serum response factor (SRF), CXC chemokine receptor 4 (CXCR4), COX-1, COX-2, cyclin D1 (Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA), extracellular signal-regulated kinase (ERK1/ERK2), phosphorylated ERK1/2, P38 mitogenic activated protein kinase (MAPK), phosphorylated P38 MAPK, phosphatidyl inositol 3-kinase (PI3K), and phosphorylated PI3K (Cell Signaling Technology, Beverly, MA, USA) overnight at 4°C, and then incubated for 1 h with secondary antibodies conjugated with peroxidase. The membrane was developed by the enhanced chemiluminescence system (Amersham Biosciences, Piscataway, NJ, USA) and was exposed to an X-ray film (Fuji Photo Film, Tokyo, Japan). Quantitation was performed via a video densitometer (Scan Marker III, Microtek, Carson, NV, USA).

Statistical analysis

The results are expressed as means ± s.e.m, n = 8–10 in each group. Differences between the means were analysed with Student's *t*-test and one-way analysis of variance when appropriate. A value of $P < 0.05$ was considered statistically significant.

Results

Effects of omeprazole on RGM-1 cell proliferation

Treatment with omeprazole (10^{-8}, 10^{-6} and 10^{-4} M) for 12 and 24 h did not significantly increase the number of RGM-1 cells when compared with the vehicle control (Table 1). When analysed further by flow cytometry, omeprazole treatment for 12 and 24 h did not substantially increase the cells in S phase as compared with the control group (Table 1). The MTT test showed that omeprazole treatment for 24 h did not produce significant changes in cell viability (Table 1). Indometacin (10^{-5} M) treatment for 24 h did not produce significant changes in cell viability when compared with the vehicle control (data not shown).

Effect of omeprazole on RGM-1 cell migration

Omeprazole treatment (10^{-8}, 10^{-6} and 10^{-4} M) promoted cell migration when compared with the vehicle control, and reached a significant difference in the higher concentration groups (10^{-6} and 10^{-4} M) after 24 h treatment (Figure 1). Indometacin (10^{-5} M) significantly decreased cell migration when compared with that in the vehicle control after 24, 36 and 48 h treatment. Co-treatment with omeprazole (10^{-4} M) and indometacin (10^{-5} M), but not 10^{-6} M omeprazole and 10^{-5} M indometacin, for 24, 36 and 48 h, reversed the inhibitory effects of indometacin on RGM-1 cell migration (Figure 2).

Protein expression after omeprazole treatment

Omeprazole treatment (10^{-8}, 10^{-6} and 10^{-4} M) for 10 min, 30 min, 1, 3, 6 and 12 h did not increase protein expression of EGF, bFGF, VEGF, SRF, CXCR4, COX-1, COX-2 and cyclin D1 when compared with the vehicle controls (data not shown). Omeprazole treatment (10^{-8}, 10^{-6} and 10^{-4} M) for 10 min, 30 min, 1, 3, 6 and 12 h also did not increase the protein expression of signal transduction pathways, including ERK1/2, phosphorylated ERK1/2, P38 MAPK, phosphorylated

Table 1 Effects of omeprazole on proliferation and viability of RGM-1 cells

Treatment	Cell count (10^5 mL^{-1})		Cells in S phase of cell cycle (% change from control)		Cell viability (% change from control)
	12 h	24 h	12 h	24 h	24 h
Control (vechicle)	25 ± 1	31 ± 1	100 ± 3%	100 ± 3%	100 ± 3%
Omeprazole 10^{-8} M	26 ± 2	32 ± 1	100 ± 2%	103 ± 2%	101 ± 4%
Omeprazole 10^{-6} M	25 ± 1	31 ± 1	101 ± 3%	101 ± 3%	103 ± 2%
Omeprazole 10^{-4} M	24 ± 1	30 ± 1	98 ± 4%	100 ± 3%	100 ±3%

Cells were counted using a haemocytometer. The S phase of the cell cycle of RGM-1 cells was analysed by flow cytometry and data were analysed using ModFit and CellQuest software. Cell viability was measured using the 3-(4,5-dimethyl-thiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) reduction method. Value are means ± s.e.m. for 8 samples per group. No significant difference was noted between groups.

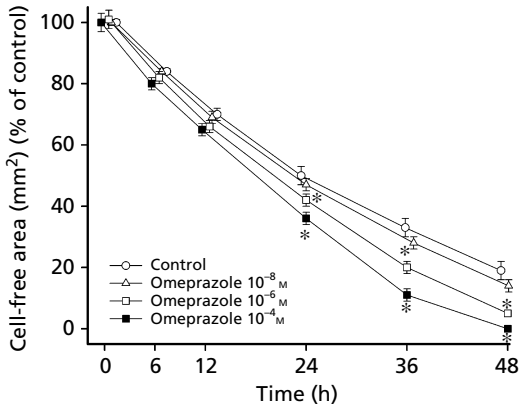

Figure 1 Effects of omeprazole on cell migration in RGM-1 cells. An artificial wound was made after cells reached confluence. Cells were incubated with vehicle control (0.5% dimethylsulfoxide) or omeprazole (10^{-8}, 10^{-6} and 10^{-4} M) for 48 h. The size of the cell-free area was monitored at time 0, 6, 12, 24, 36 and 48 h, using a digital image processor connected to a microscope. The areas were calculated with an image analysing program. Values are mean ± s.e.m. for 10 samples per group. *$P < 0.05$, significantly different compared with the control.

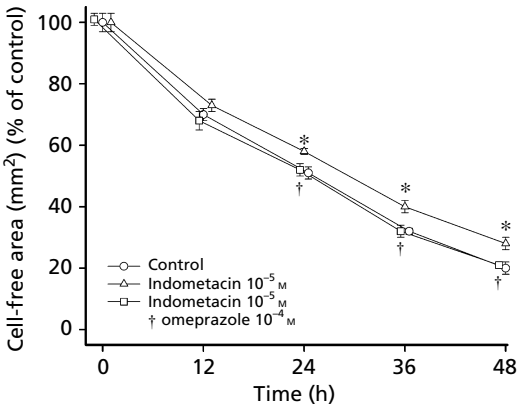

Figure 2 Effects of indometacin and omeprazole combined with indometacin on cell migration in RGM-1 cells. An artificial wound was made after cells reached confluence. Cells were incubated with vehicle control (0.5% dimethylsulfoxide, 0.02% ethanol), indometacin (10^{-5} M) or omeprazole (10^{-4} M) combined with indometacin (10^{-5} M) for 48 h. The size of the cell-free area was monitored at time 0, 12, 24, 36 and 48 h using a digital image processor connected to a microscope. The areas were calculated with an image analysing program. Values are mean ± s.e.m. for 10 samples per group. *$P < 0.05$, significantly different compared with the control group. †$P < 0.05$, significantly different compared with the indometacin group.

P38MAPK, PI3K and phosphorylated PI3K, when compared with the vehicle controls (data not shown).

Discussion

This study demonstrates for the first time that omeprazole promotes RGM-1 cell migration and abolishes the inhibitory effect of indometacin on cell migration under acid-independent conditions. The findings suggest a novel therapeutic potential of PPIs in promoting mucosal restitution both in the basal condition and after the adverse actions of indometacin, one of the most potent NSAIDs used in clinical practice.

The concentration of omeprazole in the plasma of patients is approximately 1 μM after an oral dose of 20 mg omeprazole twice daily and it can elevate to 10 μM after intravenous administration of 40 mg omeprazole (Cederberg et al 1992; Suzuki et al 1996). We believe that the promotion of gastric epithelial cell migration by omeprazole is chiefly under the systemic effect of omeprazole via continuous blood supply to the gastric mucosa and is partially under the topical effect of omeprazole in the gastric lumen. In addition, the gastric pH after omeprazole treatment may be closer to neutral pH. Therefore, the effect of omeprazole (1 and 100 μM) on epithelial cell migration resembles the real situation that may happen in clinical conditions.

The study showed that omeprazole did not increase RGM-1 cell proliferation and did not activate some signal transduction pathways and growth factor expression, such as ERK1/2, P38MAPK, PI3K, EGF, bFGF and COX-2, which are important for gastric epithelial cell proliferation (Terada et al 1999; Milani & Calabro 2001; Tarnawski et al 2001; Luo et al 2003; Tarnawski 2005). These findings also imply that the clinical use of PPIs is relatively safe against gastric carcinogenesis because of its non-mitogenic role in gastric epithelial cell proliferation. However, further studies are needed to determine if PPIs can modulate cell proliferation in human gastric cancer cells.

In general, cell migration requires cytoskeletal rearrangement: actin microfilament assembly, stress fiber formation, as well as rearrangement of focal adhesion complex attachments to the extracellular matrix (Pai et al 2001). Growth factors such as EGF, bFGF, and hepatocyte growth factor, as well as cytokines such as tumour necrosis factor-α, interleukin-1β and interleukin-8, are reported to activate cell migration (Yoo 2002; Tarnawski 2005). Our study showed that indometacin inhibited RGM-1 cell migration, which was consistent with the finding of Netzer et al (2003). Our data further showed that omeprazole promoted basal epithelial cell migration and reversed the inhibitory effect of indometacin on cell migration without increasing the expression of some of the motogenic factors such as bFGF and COX-2, nor activating motogenic pathways such as phosphorylated forms of ERK1/2 and P38MAPK (Milani & Calabro 2001; Tarnawski et al 2001; Tarnawski 2005). Future studies are needed to elucidate other possible mechanisms or chemokine gene expression regarding the promoting action of omeprazole on gastric epithelial cell migration.

Conclusions

PPIs are effective in preventing and treating NSAID-induced gastric ulcers. Our study showed that omeprazole, a type of PPI, promotes rat gastric epithelial cell migration and abolishes indometacin-associated inhibitory effects under acid-independent conditions. The findings reveal a novel therapeutic action of PPIs in modifying the delay of ulcer healing and the prevention of mucosal injury induced by NSAIDs.

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