Novel action of gastric proton pump inhibitor on suppression of *Helicobacter pylori* induced angiogenesis

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Background: Although activation of mitogen activated protein kinases (MAPKs) by *Helicobacter pylori* infection is associated with induction of host angiogenesis, which may contribute to *H pylori* associated gastric carcinogenesis, the strategy for its prevention has not been identified. As we previously reported a strong inhibitory action of gastric proton pump inhibitors (PPIs) on MAPK extracellular signal regulated kinase (ERK)1/2 phosphorylation, we investigated whether PPIs could suppress the *H pylori* induced angiogenesis via inhibition of MAPK ERK1/2.

Methods: To address the relationship between *H pylori* infection and angiogenesis, comparative analysis of density of CD34⁺ blood vessel was performed in tissues obtained from 20 *H pylori* positive gastritis and 18 *H pylori* negative gastritis patients. Expression of hypoxia inducible factor 1 (HIF-1 α) and vascular endothelial growth factor (VEGF) was tested by reverse transcription-polymerase chain reaction and secretion of interleukin 8, and VEGF was measured by ELISA. To evaluate the direct effect of *H pylori* infection on the tubular formation of human umbilical vein endothelial cells (HUVEC), an in vitro angiogenesis assay was employed. Activation of MAPK and nuclear factor κ B (NF κ B) was detected by immunoblotting.

Results: *H pylori* positive gastritis patients showed a higher density of CD34⁺ blood vessels (mean 40.9 (SEM 4.4)) than *H pylori* negative gastritis patients (7.2 $\pm$ 0.8), which was well correlated with expression of HIF-1 α . Conditioned media from *H pylori* infected gastric epithelial cells directly induced tubular formation of HUVEC and the increase of in vitro angiogenesis was suppressed by PPI treatment. Infection of *H pylori* significantly upregulated expression of HIF-1 α and VEGF in gastric epithelial cells and expression of proangiogenic factors was mediated by MAPK activation and partially responsible for NF κ B activation. PPIs effectively inhibited the phosphorylation of MAPK ERK1/2 that is a principal signal for *H pylori* induced angiogenesis.

Conclusions: The fact that PPIs could downregulate *H pylori* induced angiogenesis indicates that antiangiogenic treatment using a PPI could be a promising protective therapeutic approach for *H pylori* associated carcinogenesis.

That chronic persistent gastric inflammation associated with *Helicobacter pylori* may play a crucial role in either the development or progression of gastric cancers has been generally agreed^{1–3} but the exact molecular mechanisms of how longstanding *H pylori* infection can induce cancer and make the procancer microenvironment favourable for the survival of tumour cells have not yet been clearly identified. The mechanisms fostering the neoplastic process of *H pylori* infection have been revealed to include: (1) induction of neoplastic mutation by a considerable burden of oxidative stress^{4,5}; (2) imbalance between cell proliferation and apoptosis^{6,7}; (3) production of proteases and growth factors providing the environment for cell migration^{8–10}; and (4) induction of host angiogenesis.^{11–15}

Among the diverse host cellular responses related to *H pylori* associated inflammation or carcinogenesis, some investigators reported that angiogenic growth factors induced by *H pylori* might be primarily important.^{11–15} Kitadai and colleagues¹¹ and Cox and colleagues¹² found that *H pylori* infection induced several angiogenic factors and proteases, such as interleukin 8 (IL-8), vascular endothelial growth factor (VEGF), angiogenin, urokinase-type plasminogen activator, and metalloprotease 9 using high throughput technology of cDNA microarray analysis. Strowski and colleagues¹³ also reported that *H pylori* stimulated host VEGF gene expression via a mitogen activated protein kinase

(MAPK) pathway. These data imply that *H pylori* are capable of inducing host angiogenesis, which may play a critical role in the development and progression of gastric cancer. However, trials documenting the precise mechanism and preventive therapeutic approaches have not been performed.

The H⁺/K⁺-ATPase of gastric parietal cells exchanges luminal K⁺ for cytoplasmic H⁺ and is the enzyme primarily responsible for gastric acidification.^{16,17} The enzyme consists of two subunits, a 114 kDa α subunit and a 35 kDa β subunit. The α subunit containing ATP and cation binding sites carries out the catalytic and transporting function of the proton pump. The heavily glycosylated β subunit is required for endocytic retrieval of the H⁺/K⁺-ATPase from the canalicular membranes and is also essential for protecting the proton pump from the acid milieu environment. As abnormally controlled gastric acid causes several gastrointestinal acid related diseases, including gastro-oesophageal reflux disease, gastric ulcer, duodenal ulcer, and Barrett's oesophagus,

Abbreviations: PPI, proton pump inhibitor; PPZ, pantoprazole; HIF-1 α , hypoxia inducible factor 1; VEGF, vascular endothelial growth factor; IL-8, interleukin 8; MAPKs, mitogen activated protein kinases; ERK, extracellular signal regulated kinase; NF κ B, nuclear factor κ B; RT-PCR, reverse transcription-polymerase chain reaction; HUVEC, human umbilical vein endothelial cells; PBS, phosphate buffered saline; CFU, colony forming units; PDTC, 1-pyrrolidinedithiocarbamic acid; ELISA, enzyme linked immunosorbent assay

gastric proton pump inhibitors (PPIs) have been developed for the treatment of these acid related diseases.^{18–20} Among these, pantoprazole, a substituted 2-pyridyl methyl/sulfinyl benzimidazole derivative, is a prodrug requiring protonation for functional activation under acidic conditions, accumulates selectively in acidic gastric luminal space, and ultimately inhibits acid secretion by covalent binding with cysteine residues in the α subunit of H^+/K^+ -ATPase.^{18–20} The last decade has seen standardisation of the treatment regimens for *H. pylori* eradication, with the use of triple therapy consisting of a PP and two antibiotics, mainly clarithromycin and amoxicillin. Blockage of gastric acid secretion by PPIs contributes towards eradication of *H. pylori* via the rising pH of the gastric lumen. Appropriately high pH values increase antimicrobial susceptibility of *H. pylori* because the minimum inhibitory concentration of most antibiotics against *H. pylori* is very dependent on the pH of the environment.^{21–26}

Previously, we found that PPIs could exercise selective induction of apoptosis in gastric cancer cells, which was due to a significant inhibitory action of PPIs on MAPK activation.²⁷ As Strowski and colleagues¹³ reported that *H. pylori* stimulated host VEGF gene expression via the MAPK pathway, we hypothesised that PPIs could exert antiangiogenesis actions through MAPK inhibition in *H. pylori* induced angiogenesis. Here, we have found that infection with *H. pylori* significantly upregulated angiogenesis of the gastric mucosa by strong induction of proangiogenic factors, including IL-8, hypoxia inducible factor 1 (HIF-1 α), and VEGF and, remarkably, angiogenesis induced by *H. pylori* was attenuated by PPI treatment.

MATERIALS AND METHODS

Tissue samples

Biopsied samples were obtained from five patients (mean age 48 years) with functional dyspepsia without *H. pylori* infection, 20 patients (mean age 55 years) with chronic active *H. pylori* positive gastritis, and 18 patients (mean age 54 years) with *H. pylori* negative gastritis induced mostly by non-steroidal anti-inflammatory drugs or other causes during gastroscopy. The presence of *H. pylori* was determined using the following tests: haematoxylin-eosin staining and Giemsa staining of biopsied tissues, rapid urease test, and urea breath test. When all of the above were negative, the case was defined as *H. pylori* negative and if more than two of these tests were positive, the case were defined as *H. pylori* positive. Gastritis was evaluated histologically and scored according to a modified Sydney classification²⁸; two different pathologists scored the degree of gastritis independently. Informed written consent was obtained from patients and the study was approved by Institutional Review Board.

Immunohistochemistry for counting vessels in the gastric mucosa

Immunohistochemical staining of CD34⁺ endothelial cells was performed to analyse the degree of angiogenesis in the gastric mucosa of gastritis patients. For immunohistochemical detection, 10% buffered formalin fixed paraffin embedded sections were deparaffinised, rehydrated, and then boiled in 100 mM Tris buffered saline (pH 7.6) with 5% urea in an 850 W microwave oven for five minutes, followed by two more treatments of five minutes each. Then sections were stained with Histostain-Plus kit (Zymed Laboratories Inc., San Francisco, California, USA) according to the manufacturer's instructions. Primary antibodies against the CD34 endothelial cell marker were purchased from Novocastra Laboratories (clone QBEnd/10; UK). Sections were counterstained with haematoxylin. CD34 positive blood vessels were counted on three separate sites

($\times 100$ magnified field) and presented as mean (SEM) of 20 *H. pylori* positive and 18 *H. pylori* negative cases.

Cell culture, bacteria strain, and reagents

Human gastric epithelial AGS cells were cultured in RPMI 1640 medium (Gibco BRL, Grand Island, New York, USA) containing 10% fetal bovine serum and 100 U/ml penicillin in a humidified 5% CO₂ atmosphere. Human umbilical vein endothelial cells (HUVEC) were obtained by collagenase treatment of umbilical cord veins, as previously described.²⁹ Cells were cultured on gelatin coated dishes and propagated in RPMI 1640 medium supplemented with 20% bovine calf serum, 90 μ g/ml heparin (Sigma Chemical Co, St Louis, Missouri, USA), and 50 μ g/ml endothelial cell growth factor. A *cagA*⁺ and *vaca*⁺ standard strain of *H. pylori* (ATCC 43504) was obtained from the American Type Culture Collection (ATCC, Manassas, Virginia, USA). *H. pylori* were recovered from frozen stock by seeding on a blood agar plate including 7% sheep blood at 37°C for five days under microaerophilic conditions (5% O₂, 10% CO₂) generated with campy pouch (Becton Dickinson Microbiology Systems, Sparks, Maryland, USA). For inoculation of the bacteria, *H. pylori* were resuspended in phosphate buffered saline (PBS) to an A₄₅₀ of 1.2 units, which corresponds to a bacterial concentration of 5×10^8 colony forming units (CFU)/ml, and cocultured with AGS cells at a concentration of 5×10^7 CFU/ml.

A solution of pantoprazole (PPZ) was obtained from Altana Pharma AG (Konstanz, Germany). PD098059 (50 μ M, extracellular signal regulated kinase (ERK)1/2 inhibitor; Cell Signaling Technology, Beverly, Massachusetts, USA), SB203580 (10 μ M, p38 inhibitor; Cell Signaling Technology), 1-pyrrolidinedithioic acid (PDTIC 100 μ M, ammonium salt, nuclear factor κ B (NF κ B) inhibitor; Calbiochem, La Jolla, California, USA), and BAY11-7082 (5 μ M, NF κ B inhibitor; Calbiochem) were used in the cell culture experiments. Briefly, to evaluate the effect of these inhibitors or PPIs, they were preincubated with AGS cells for eight hours, washed with PBS, and inoculated with *H. pylori*.

Western blot analysis

Human gastric epithelial AGS cells (1×10^7 cells/100 mm² culture dish) were incubated with 0, 100, 200, or 400 μ M PPI for eight hours, washed with PBS three times, and then inoculated with *H. pylori* (5×10^7 CFU/ml) for 15 minutes (western blotting for ERK1/2) or two hours (western blotting for NF κ B). Cells were resuspended in lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, and protease inhibitor cocktail; Roche, Mannheim, Germany). The suspension was sonicated for approximately 30 seconds and centrifuged at 15 000 *g* for 30 minutes. For documenting NF κ B activation, nuclear/cytosolic fractionation was performed using NE-PER Nuclear and Cytoplasmic Extraction Kit (Pierce, Rockford, Illinois, USA) following the manufacturer's protocol. Isolated protein (20 μ g) was subjected to western blotting. Proteins were extracted from the cells, electrophoresed on 12% sodium dodecyl sulphate-polyacrylamide gels, and transferred to PVDF membranes using a semidry transfer system (Hoeffer Pharmacia Biotech, San Francisco, California, USA). Membranes were blocked in 5% non-fat dry milk and probed with 1:1000 dilution of specific antibodies corresponding to phospho-ERK (E-4), total ERK (K-23), NF κ B p65 (F-6), or α -tubulin (TU-02); all antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA).

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated from cells with appropriate treatment using TRIzol reagent (Life Technologies, Milan, Italy), and

Figure 1 Immunohistochemical staining of the CD34 endothelial cell marker. (A) Immunohistological analysis of CD34 positive blood vessels in gastric biopsy specimens of *Helicobacter pylori* negative (a, 100 $\times$; c, 200 $\times$) and *H. pylori* positive (b, 100 $\times$; d, 200 $\times$) gastritis. Specimens obtained from *H. pylori* negative gastritis or *H. pylori* positive gastritis during endoscopic examination were used for immunohistological analysis with anti-CD34 antibodies. CD34 $^{+}$ blood vessels were stained by a dark red colour (arrow), counterstained with haematoxylin showing a blue colour. (B) Mean number of blood vessels stained with CD34. Number of blood vessels with equal dimensions were counted in triplicate for each sample and are represented as means (SD). There was a statistically significant difference between the two groups ($p < 0.01$). *Hp*, *Helicobacter pylori*.

2 μ g total RNA were reverse transcribed according to the manufacturer's instructions (M-MLV reverse transcriptase; Promega, Madison, Wisconsin, USA). PCR was performed using the Premix Ex Taq kit (Takara, Chiba, Japan) with specific primers as follows: 5'-CTC AAA GTC GGA CAG CCT

CA -3' and 5'-CCC CGC AGT AGG TTT CTG CT-3' for *HIF-1 α* ; 5'-TCG GGC CTC CGA AAC CAT G-3' and 5'-GGT TCC CGA AAC CCT GAG G-3' for *VEGF*; and 5'-TTG TTG CCA TCA ATG ACC CC-3' and 5'-TGA CAA AGT GGT CGT TGA GG-3' for *GAPDH*. The PCR reaction was carried out for 28 thermal

Figure 2 Expression of hypoxia inducible factor 1 (HIF-1 α) mRNA in human gastric mucosa. (A) Reverse transcription-polymerase chain reaction of HIF-1 α was done with total RNA isolated from *Helicobacter pylori* (*Hp*) negative gastritis ($n = 5$), *H. pylori* positive gastritis ($n = 5$), and normal stomachs ($n = 5$). (B) Relative expression of HIF-1 α mRNA is represented as mean intensity/mm ^{2} . HIF-1 α expression was significantly higher in gastric mucosa of *H. pylori* positive gastritis than in *H. pylori* negative gastritis in spite of similar expression of GAPDH ($p < 0.01$).

cycles of 94°C for one minute at 55°C (for HIF-1 α and GAPDH) or 60°C (for VEGF) for one minute, and 72°C for one minute. The product was resolved on 1% agarose gel and stained with ethidium bromide.

Enzyme linked immunosorbent assay (ELISA)

Immunoreactive human IL-8 and VEGF were measured in culture supernatants of AGS cells using enzyme linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions (HyCult Biotechnology, Uden, the Netherlands). AGS cells were grown in six well cell culture dishes, incubated in the presence or absence of PPI for eight hours, and after washing with PBS, cocultured with *H. pylori* for various times. Culture supernatant (200 μ l) was used for analysis of IL-8 and VEGF production.

In vitro angiogenesis assay

In vitro angiogenesis assay was slightly modified from Kitadai and colleagues.¹¹ Briefly, AGS cells (1×10^7 cells/100 mm² culture dish) were incubated with 0, 100, 200, or 400 μ M PPI for eight hours, washed with PBS three times, and then inoculated with *H. pylori* (5×10^7 CFU/ml) for 24 hours. The cell culture supernatant was harvested and centrifuged at 5000 *g* for 30 minutes. Conditioned media was prepared by 1:1 dilution of the culture supernatant with HUVEC endothelial cell medium. The conditioned media were filtered through 0.4 μ M pore filters (Millipore, Boston, Massachusetts, USA) to remove *H. pylori* and then the media were added to HUVEC culture and changed every three days. After nine days, the HUVEC were observed for tubular formation under microscopy and confirmed expression of the endothelial cell marker, CD31, by immunocytofluorescence staining. HUVEC were fixed in ice cold methanol for five minutes, frozen at -20°C, and treated with 1% Triton X-100/PBS for 10 minutes at room temperature. Cells were blocked with 5% bovine serum albumin for 30 minutes and probed with anti-CD31 antibodies (1:100 diluted in 5% bovine serum albumin; Santa Cruz Biotechnology) for two hours. Cy3 conjugated secondary antibodies were used to visualise under

a inverted fluorescence microscope (Olympus, IX71, Tokyo, Japan).

Statistics

All values are expressed as mean (SEM) and the Mann-Whitney U test and Friedman ANOVA test were used for statistical calculations.

RESULTS

Distinct expression of CD34⁺ blood vessels between *H. pylori* positive and negative gastritis patients

Stomach tissue samples were obtained from gastritis patients during endoscopy examination and were evaluated histologically by haematoxylin-eosin staining. Finally, we choose 20 *H. pylori* positive gastritis and 18 *H. pylori* negative gastritis cases with a similar degree of gastritis, scored according to the modified Sydney classification,²⁸ as the degree of gastric inflammations itself can affect angiogenesis. Immunohistochemical staining using antibodies against the CD34 endothelial cell marker was performed to evaluate the difference in angiogenesis according to *H. pylori* infection. The results showed that patients with *H. pylori* positive gastritis (fig 1A (b, d)) showed significantly higher expression of CD34 positive blood vessels in the gastric mucosa layer than that of patients with *H. pylori* negative gastritis (fig 1A (a, c)). The number of blood vessels was counted in three sites, for each specimen, with equal dimensions, and mean levels are shown in fig 1B. While *H. pylori* negative gastritis samples had a mean of 7.2 (SEM 0.8) blood vessels per $\times 100$ magnified field, *H. pylori* positive gastritis samples had a significant increased number (mean 40.9 (SEM 4.4)) of blood vessels ($p < 0.01$). Moreover, blood vessels observed in cases with *H. pylori* positive gastritis (fig 1A (b, d), arrow) were thicker and larger than those of *H. pylori* negative cases (fig 1A (a, c), arrow). Interestingly, blood vessels were found more abundantly in the mesenchymal stromal layer below the mucosa layer but the number of blood vessels in the stromal layer was not different between *H. pylori* positive gastritis and *H. pylori* negative gastritis cases, suggesting that *H. pylori* infection may

Figure 3 In vitro angiogenesis assay. AGS cells (1×10^7 cells/100 mm² culture dish) were incubated with 0, 200, or 400 μ M proton pump inhibitor (PPI) for eight hours, washed with phosphate buffered saline three times, and inoculated with *Helicobacter pylori* (5×10^7 CFU/ml) for 24 hours. Conditioned media were prepared from 1:1 dilution of the cell culture supernatant and the human umbilical vein endothelial cell (HUVEC) medium. Conditioned media were filtered through a 0.4 μ M pore filter to remove *H. pylori* and then added to the HUVEC culture which was changed every three days. After nine days, HUVEC were observed in a tubular formation under microscopy (A–E) and expression of the endothelial cell marker, CD31, was confirmed by immunocytofluorescence staining (F–J). (A, F) Control HUVEC cells; (B, G) HUVEC cells incubated with conditioned media of *H. pylori* infected AGS; (C, H) HUVEC cells incubated with conditioned media of 400 μ M PPI treated AGS; (D, I) HUVEC cells incubated with conditioned media of 200 μ M PPI/*H. pylori* infected AGS; (E, J) HUVEC cells incubated with conditioned media of 400 μ M PPI/*H. pylori* infected AGS.

Figure 4 Release and expression of vascular endothelial growth factor (VEGF) and interleukin 8 (IL-8) from *Helicobacter pylori* infected AGS cells. (A) Production of VEGF (top) and IL-8 (bottom) was measured by ELISA with the culture supernatant of AGS cells infected with *H. pylori* for the indicated times. (B) Induction of hypoxia inducible factor 1 (HIF-1α), VEGF, and IL-8 mRNA by *H. pylori* infection was tested by reverse transcription-polymerase chain reaction analysis in AGS cells incubated with the bacterium for the indicated times. (C) Relative band intensity is presented as fold ratio.

be associated with induction of angiogenesis in *H. pylori* infected gastric mucosa.

We then evaluated if there were any differences in expression of HIF-1α, the potent angiogenic transcriptional factor (fig 2). As a control, we used gastric biopsies obtained from five cases diagnosed with functional dyspepsia with no significant abnormal gastroscopic findings and no *H. pylori* infection. Compared with HIF-1α mRNA from five normal stomachs, expression was not altered in five *H. pylori* negative gastritis but was significantly increased in *H. pylori* positive gastritis ($p < 0.01$), suggesting that HIF-1α is responsible for *H. pylori* induced angiogenesis (fig 2B).

Suppression of *H. pylori* induced in vitro angiogenesis by gastric PPI

To prove a direct effect of *H. pylori* infection on angiogenesis, we performed an in vitro angiogenesis assay. Conditioned media obtained from *H. pylori* infected AGS cells were added to HUVEC culture flasks and morphological changes in the endothelial cells were observed. After nine days, HUVEC became long in shape and formed a tubular structure (fig 3B) compared with conditioned media of non-*H. pylori* infected AGS (fig 3A). CD31 immunofluorescence staining showed a dense intensity of CD31 molecules in HUVEC cells incubated with the culture supernatants of *H. pylori* infected AGS (fig 3G) while control media obtained from non-*H. pylori* infected AGS stimulated neither tubular formation of HUVEC nor expression of the CD31 endothelial cell marker (fig 3A, F).

Results of in vitro angiogenesis assay strongly suggested that *H. pylori* infection stimulated infected gastric epithelial cells to secrete proangiogenic factors which induce growth and differentiation of endothelial HUVEC. Interestingly, pretreatment with PPI (200 μM or 400 μM, for eight hours) on AGS cells prior to *H. pylori* inoculation significantly inhibited tubular formation (fig 3D, E) and CD31 expression of endothelial HUVEC (fig 3I, J). However, no significant

changes were noted in HUVEC incubated with 400 μM PPI alone (fig 3C, H), suggesting that PPI itself did not influence tubular formation of HUVEC. The data clearly indicate that PPI suppressed *H. pylori* induced in vitro angiogenesis, suggesting that antiangiogenic treatment with PPI could be a promising therapeutic approach for *H. pylori* associated carcinogenesis.

Production of proangiogenic factors from *H. pylori* infected gastric epithelium and its inhibition by PPI

Following *H. pylori* infection, AGS cells significantly secreted VEGF and IL-8, well characterised as proangiogenic factors, in a time dependent manner (fig 4A). Maximal induction of IL-8 (mean 1019 (SEM 278) pg/ml) and VEGF (1597 (94) pg/ml) was observed after 24 hours of inoculation (fig 4A). We also examined mRNA expression of these angiogenic factors using RT-PCR analysis (fig 4B). Expression of VEGF mRNA, one of the HIF-1α target genes, was induced after 16 hours of *H. pylori* infection, showing the correlation with HIF-1α expression (fig 4B). IL-8 mRNA was also significantly induced after *H. pylori* infection (fig 4B). All of these results suggest that synthesis of angiogenic growth factors is stimulated by *H. pylori* infection in gastric epithelial cells, which could induce proliferation and differentiation of endothelial cells.

Because PPI showed a strong antiangiogenic action in the in vitro angiogenesis assay (fig 3), we measured the effect of PPI on expression of these angiogenic factors (fig 5). Secretion of IL-8 in the supernatants of *H. pylori* infected AGS cells was found to be remarkably suppressed after PPI treatment in a dose dependent manner (fig 5A). Following eight hours of infection with *H. pylori*, IL-8 production increased up to 870 pg/ml but this increment in IL-8 production was significantly attenuated by PPI pretreatment. Pretreatment with PPI showed a considerable regulatory effect on *H. pylori* mediated VEGF synthesis (fig 5A). Suppression of these angiogenic factors by PPI was evidenced

Figure 5 (A) Effects of proton pump inhibitor (PPI) on expression of angiogenic growth factors interleukin 8 (IL-8), hypoxia inducible factor 1 (HIF-1 α), and vascular endothelial growth factor (VEGF). To examine the inhibitory effect of PPI on *Helicobacter pylori* induced angiogenic growth factor expression, AGS cells (1×10^7 cells/100 mm² culture dish) were incubated with 0, 50, 100, 200, or 400 μ M PPI for eight hours, washed with phosphate buffered saline three times, and inoculated with *H. pylori* (5×10^7 CFU/ml) for 16 hours. Production of IL-8 (top) and VEGF (bottom) was measured in culture supernatants of the cells by ELISA. (B) Total RNA extracted from cells was used in the reverse transcription-polymerase chain reaction analysis of HIF-1 α and VEGF.

by transcriptional inhibition of the genes (fig 5B). At 400 μ M of PPI, expression of VEGF and HIF-1 α seemed to decline relevant to that of control AGS cells.

Expression of *H. pylori* induced angiogenic factors is mediated by activation of ERK1/2

As VEGF and IL-8 expression was found to be regulated by MAPK and NF κ B signalling, we assessed the involvement of MAPK ERK1/2 and NF κ B on *H. pylori* induced angiogenesis using specific inhibitors (fig 6). PD098059 (50 μ M), one of the ERK inhibitors, strongly inhibited *H. pylori* induced HIF-1 α and VEGF expression, and SB203580 (10 μ M), a p38 inhibitor, was also able to inhibit expression of these angiogenic factors. PDTC (ammonium salt, 100 μ M) a

Figure 6 Involvement of mitogen activated protein kinase and nuclear factor κ B (NF κ B) in *Helicobacter pylori* induced mRNA expression of hypoxia inducible factor 1 (HIF-1 α) and vascular endothelial growth factor (VEGF). Prior to inoculation with *H. pylori*, AGS cells were treated with each inhibitor (50 μ M PD098059, 10 μ M SB203580, 100 μ M PDTC, or 5 μ M BAY11-7082 (BAY)) for eight hours, and their effects on *H. pylori* induced HIF-1 α and VEGF expression were evaluated by reverse transcription-polymerase chain reaction. PD, PD098059, extracellular signal regulated kinase (ERK)1/2 inhibitor; SB, SB203580, p38 inhibitor; PDTC, 1-pyrrolidinedithiocarbamate, ammonium salt, NF κ B inhibitor; BAY, BAY11-7082, NF κ B inhibitor.

NF κ B inhibitor, potently suppressed HIF-1 α and VEGF expression induced by *H. pylori*. BAY11-7082 (5 μ M) reversibly increased expression of the genes. These data suggest that *H. pylori* induced VEGF induction was mediated via the MAPK pathway, and partially by the NF κ B pathway.

PPI disturbs *H. pylori* induced signalling for angiogenesis via inactivation of MAPK ERK1/2

Based on the previous findings that *H. pylori* infection stimulated the synthesis of angiogenic factors via MAPK ERK activation (fig 6) and that the anticancer action of PPI is fundamentally attributable to inhibition of phosphorylation of MAPK ERK1/2,²⁷ we evaluated whether the antiangiogenic activity of PPI is caused by block of ERK activation. Western blot analysis with phospho-ERK antibodies was performed to determine the influence of PPI on MAPK ERK1/2 activation related to *H. pylori* induced angiogenesis (fig 7A, B). The ERK inhibitor, PD098059, and the p38 inhibitor, SB203580, decreased phosphorylation of ERK1/2. Interestingly, PPI completely attenuated phosphorylation of ERK1/2, showing stronger inhibitory activity than the ERK1/2 inhibitor PD098059 (fig 7A). These inhibitory actions of PPI against ERK phosphorylation were dose dependent (fig 7B) and maximal inhibitory activity was seen after four hours (data not shown). However, PPI did not have any effect on nuclear translocation of NF κ B, suggesting that the antiangiogenic effect of PPI seems to be independent of suppression of NF κ B (fig 7C). In summary, angiogenesis induced by *H. pylori* was attenuated by PPI treatment, which was caused by inactivation of the MAPK pathway, one of principal signals for *H. pylori* induced angiogenesis.

DISCUSSION

After *H. pylori* infection, the signal transduction enzymes, MAPK ERK1/2 and NF κ B, are activated and these molecules are responsible for transcriptional activation of angiogenic growth factors, including IL-8, HIF-1 α , and VEGF. Increase in *H. pylori* induced angiogenic factors stimulates the recruitment and activation of endothelial cells in the gastric mucosa, resulting in significant neovascularisation of the gastric mucosal layer which can provide a vulnerable and fertile environment for carcinogenesis. Chronic gastric

Figure 7 Deactivation of mitogen activated protein kinase (MAPK) extracellular signal regulated kinase (ERK)1/2 signalling with proton pump inhibitor (PPI). (A) To compare the inhibitory effect of MAPK inhibitors and PPI on phosphorylation of MAPK ERK1/2, AGS cells were treated with 50 μM of the ERK inhibitor (PD098059), 10 μM of the p38 inhibitor (SB203580), or 200 μM PPI for eight hours, and then infected with *Helicobacter pylori* for 15 minutes. Proteins isolated from cells were subjected to immunoblotting with phospho-ERK antibodies. (B) Effect on phosphorylation of ERK at different concentrations of PPI. (C) Effect of PPI on *H. pylori* induced nuclear factor κB (NFκB) translocation was evaluated at different concentrations of PPI. AGS cells were treated with the indicated concentrations of PPI for eight hours and inoculated with *H. pylori* for two hours. Nuclear proteins were isolated from cells and subjected to western blotting using specific NFκB antibodies. Translocation of NFκB was deduced from NFκB in nuclear fractions compared with total NFκB. PD, PD098059 (ERK1/2 inhibitor), SB, SB203580 (p38 inhibitor).

inflammation triggered by *H. pylori* infection may predispose to the development and progression of gastric cancer. *H. pylori* induced angiogenesis might contribute to this *H. pylori* associated gastric carcinogenesis along with other carcinogenic events. In this study, for the first time, we have documented the mechanistic link between *H. pylori* infection and angiogenesis, and the inhibitory effect of PPIs on *H. pylori* induced angiogenesis. PPI treatment efficiently inhibited IL-8, VEGF, and HIF-1α expression in *H. pylori* infected gastric epithelial cells, which was due to inactivation of MAPK signalling induced by *H. pylori* infection (fig 7). Thus these findings have helped shed light on antiangiogenic treatment with PPIs, drugs popularly prescribed for gastro-oesophageal acid related diseases and *H. pylori* eradication. PPI therapy could be a promising protective therapeutic approach for *H. pylori* associated carcinogenesis.

Neovascularisation, the development of new blood vessels from existing endothelial precursors, is a general physiological

mechanism critically involved in the normal repair process and in the pathogenesis of inflammatory and ulcerative epithelial lesions as well as malignant tumour growth, and even tumour metastasis.^{30–31} Among the proangiogenic factors known, VEGF represents one of the most potent stimuli of neoangiogenesis. Although previous studies found that enhanced VEGF gene expression contributed to the healing of peptic lesions in the stomach,^{32–33} here we demonstrated that *H. pylori* infection stimulated upregulation of proangiogenic factors and increased infiltration of blood vessels in the mucosa layer, which might be positively associated with propagation of gastric inflammation as well as gastric carcinogenesis after *H. pylori* infection. Several investigations suggested that *H. pylori* infection stimulates host VEGF-A gene expression and *H. pylori* induced angiogenesis may play a critical role in the development of gastric cancer.^{34–36} Gastric adenocarcinomas frequently showed high levels of VEGF expression,^{34–35} and neutralisation of circulating VEGF with specific VEGF antibodies potentially reduced the growth of gastric cancer.³⁶ Potent stimulators of angiogenesis related to *H. pylori* infection, *H. pylori* VacA toxin,³⁷ reactive oxygen species synthesised from neutrophils or macrophages,³⁸ *CagA* pathogenicity islands,^{12–14} and lipopolysaccharide have been reported, and several pathways linking the bacterium to host angiogenesis have been revealed. A wider spectrum of genes induced by *H. pylori* in gastric epithelium was identified by high throughput analysis of cDNA microarray analysis, and most of these genes are responsible for angiogenesis and tumour invasion stimuli, such as ADAM series, IL-8, VEGF, integrins, VCAM-1, ICAM-1, E-selectin, GRO-α, and IL-6.^{12–14}

The PPI PPZ, a substituted 2-pyridyl methyl/sulfinyl benzimidazole derivative, is a prodrug requiring protonation under acidic conditions for functional activation, accumulates selectively in the acidic gastric luminal space, and ultimately inhibits acid secretion by covalent binding with cysteine residues on the α subunit of H⁺/K⁺-ATPase. These PPIs have been universally used with antibiotics for the eradication of *H. pylori* and several types of acid related diseases, including gastro-oesophageal reflux diseases, peptic ulcer disease, and Zollinger-Ellison syndrome.^{19–20} Increased gastric pH by PPIs stimulates resting *H. pylori* to activate metabolically and thus *H. pylori* are more susceptible to antibiotics.^{21–26} Apart from enhancing the susceptibility of *H. pylori* to antibiotics, we found, for the first time, that PPIs can directly influence host angiogenesis induced by *H. pylori*. Previously, we reported that PPIs had a strong inhibitory effect on phosphorylation of MAPK ERK1/2 and its administration showed anticancer activity in the xenograft nude mice model.²⁷ These inhibitory actions of the drug against ERK phosphorylation also involved suppression of *H. pylori* induced host angiogenesis. Blocking of H⁺ by PPIs caused an increase in extracellular pH (gastric lumen) and a decrease in intracellular pH. The increased pH of the extracellular space may improve the hypoxic microenvironment surrounding gastric epithelial cells and interrupt *H. pylori* induced intracellular signalling via inhibition of MAPK activation. Compared with inhibitors of ERK1/2 or p38, PPZ showed stronger inhibitory activities than these (fig 7), in a dose dependent manner. However, the influence of this drug on NFκB transcriptional activation was minor (fig 7).

In conclusion, we have shown that considerable angiogenic activities were stimulated in the gastric mucosa after *H. pylori* infection, for which increasing proangiogenic growth factors from gastric epithelial cells were responsible. PPIs could have significant inhibitory activities against *H. pylori* associated angiogenesis. Therefore, the current data indicate that as *H. pylori* infection causally promoted host angiogenesis, which has been attributed to either augmented inflammation or enhanced carcinogenesis, PPIs could be potentially used for inhibition of *H. pylori* provoked angiogenesis.

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