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Novel mechanism of gastric proton pump inhibitor (PPI) : Suppression of *Helicobacter-pylori* induced angiogenesis and selective induction of apoptosis in gastric cancer cells.

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김동규의 의학 석사학위를 인준함.

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ABSTRACT

**Novel Mechanism of Gastric Proton Pump (PPI) Inhibitor**: Suppression of *Helicobacter pylori* Induced Angiogenesis and Selective Induction of Apoptosis in Gastric Cancer Cells.

To survival in an ischemic microenvironment with a lower extracellular pH, ability to up-regulate proton extrusion is critical for cancer cell survival. Gastric H⁺/K⁺-ATPase exchanges luminal K⁺ for cytoplasmic H⁺ and is the enzyme primarily responsible for gastric acidification. On the basis of the fact that blocking the clearance of acidic metabolites are known to induce the cell death, we hypothesized that pantoprazole (PPZ), one of gastric H⁺/K⁺-ATPase inhibitors used frequently to treat acid-related diseases, could inhibit growth of tumor cells. And, 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.

To detect PPZ-induced apoptosis, we performed that Genomic DNA fragmentation, terminal deoxynucleotidyl transferase (Tdt)-mediated nick end labeling assay, and annexin V
staining. And mitogen-activated protein kinase activation and heat shock proteins expression were determined by immunoblot with specific antibodies. The antitumor effect of PPZ was evaluated in vivo by a xenograft model of nude mice. And, 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-1α) 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 κB was detected by immunoblotting.

After PPZ treatment, apoptotic cell death was seen selectively in cancer cells and was accompanied with extracellular signal-regulated kinase deactivation. By contrast, normal gastric mucosal cells showed the resistance to PPZ-induced apoptosis through the overexpression of anti-apoptotic regulators including HSP70 and HSP27. In a xenograft model of nude mice, administration of PPZ significantly inhibited tumorigenesis and induced large-scale apoptosis of tumor cells. And, 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±0.8), which was well correlated with expression of HIF-1α. 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-1α and VEGF in gastric epithelial cells.
and expression of proangiogenic factors was mediated by MAPK activation and partially responsible for NFκB activation. PPIs effectively inhibited the phosphorylation of MAPK ERK1/2 that is a principal signal for *H. pylori* induced angiogenesis.

In conclusion, PPZ selectively induced *in vivo* and *in vitro* apoptotic cell death in gastric cancer, suggesting that proton pump inhibitors could be used for selectively anticancer effects. And, the fact that PPZ could downregulate *H. pylori* induced angiogenesis indicates that antiangiogenic treatment using a PPZ could be a promising protective therapeutic approach for *H. pylori* associated carcinogenesis.

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Key words : Proton pump inhibitor, Apoptosis, Gastric cancer cell, H⁺/K⁺-ATPase, anticancer effect, *Helicobacter pylori*, Angiogenesis, VEGF, HIF-1α, Carcinogenesis
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I. INTRODUCTION

The H⁺/K⁺-ATPase of gastric parietal cell exchanges luminal K⁺ for cytoplasmic H⁺ and is the enzyme primarily responsible for gastric acidification (Marie et al, 2004; Scott et al, 1993; Besancon et al, 1993; Pouyet et al, 1992). 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 proton pump from the environment of acid milieu. Because abnormally controlled gastric acids secreted by H⁺/K⁺-ATPase caused several gastrointestinal acid-related diseases including gastroesophageal reflux disease, gastric ulcer, duodenal ulcer, and Barrett’s esophagus, gastric proton pump inhibitors have been developed as the treatment for these acid-related diseases (Horn et al, 2000; Sachs et al, 1997; Sachs et al, 1995; Fitton et al, 1996). Pantoprazole (PPZ) of these proton pump inhibitors, a substituted 2- pyridylmethyl/sulfinyl benzimidazole derivative, is a prodrug requiring protonation for functional activation at acidic conditions, accumulating selectively in acidic gastric luminal space and ultimately inhibiting acid secretion by the covalent binding with cystein residues in α-subunit of H⁺/K⁺-ATPase.

Besides of gastric H⁺/K⁺-ATPase, several kinds of vacuolar-type H⁺-ATPase are ubiquitously found on the membrane of various intracellular compartments of eukaryotic cells such as lysosomes, endosomes, the Golgi complex, and secretory granules (Nelson et al,
1995). Essential regulation of pH in cytoplasmic, intraorganellar, and local extracellular spaces through vacuolar-type H+-ATPase has been suggested to play an important role in the mechanism of cell survival. These facts can be credited by several studies showing that vacuolar-type H+-ATPase inhibitor, bafilomycin A or concanamycin A, strongly induced apoptotic cell death (Nishihara et al, 1995; Ishisaki et al, 1999; Ohta et al, 1998; Aiko et al, 2002).

Cell survival and cell death are tightly controlled by numerous signal enzymes and regulators such as mitogen-activated protein kinases (MAPKs) and heat shock proteins (HSPs). MAPK signal enzymes are divided into three major groups, extracellular signal-regulated kinases (ERKs), c-Jun NH$_2$-terminal kinases (JNKs)/stress-activated kinases, and p38 (Franklin et al, 2000; Johnson et al, 2002). The ERKs appear to play a crucial role in the process of extracellular signals to the nucleus leading to induction of cellular growth, proliferation, and differentiation (Ballif et al, 2001; Dent et al, 1998). Heat shock proteins, which function mainly as molecular chaperones, allow cells to adapt to their environmental changes and to survive in otherwise lethal conditions (Garrido et al, 2001; Mehlen et al, 1996; Garrido et al, 1999; Bruey et al, 2000; Mosser et al, 1997; Buzzard et al, 1998; Beere et al, 2000). Because HSPs include pro- and antiapoptotic proteins that interact with a variety of cellular proteins, the type of HSP induced and its level of expression can determine the fate of a cell in response to a death stimulus. Generally, HSP60, HCS70, and HSP90 are constitutively expressed in mammalian cells, whereas HSP70 and HSP27 are strongly induced by different stresses, such as heat, oxidative stress, or anticancer drugs. It is well known that HSP27 and HSP70 play a cytoprotective role in gastrointestinal damage.

Maintenance of intra- or extracellular pH is very much important for cell function, and cancer cells \textit{in vivo} often exist in an ischemic microenvironment with a lower extracellular pH than surrounding normal cells (Stubbs et al, 1999; Helmlinger et al, 1997; Stubbs et al, 1992; Suubbs et al, 1994; Frenzel et al, 1994; Gillies et al, 1994). The acidity in tumors is due to the increased production of acidic metabolites from rapid and large amounts of glycolysis and is provoked by the limited ability of the tumor vasculature to remove these acidic products. To overcome this hypoxic microenvironment and to prevent the accumulation of the increased acidic metabolites, the ability to dispose of intracellular protons is critical for cancer cell survival (Mccoy et al, 1995; Tannock et al, 1989; Martinez-et al, 1993; Lee et al, 1998). These findings support the rationale of the present study that the inhibition of proton extrusion might be more susceptible or vulnerable to cell death of cancer cells than normal cells.

In this study, we have demonstrated for the first time that PPZ, the H$^+$/K$^+$-ATPase inhibitor, induced apoptosis selectively in gastric cancer cells and significantly inhibited tumorigenesis in a tumor xenograft model. We also documented the mechanism of the selectivity of this proton pump inhibitor on apoptosis of cancer cells. Our novel finding suggests that proton pump inhibitors could be considered as the selective anticancer agents in gastric cancer.

And another PPZ study, we investigated that PPZs could exert angiogenesis actions through MAPK inhibitions in \textit{H.pylori} induced angiogenesis. 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 (Peek et al, 2002; Stolte et al, 2002; Scheiman et al, 1999) but the exact molecular mechanisms of how longstranding *H. pylori* infection can induce and make the procancer microenvironment favourable for the survival of tumor 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 (Touati et al, 2003; Bagchi et al, 1996); (2) imbalance between cell proliferation and apoptosis (Maeda et al, 2002; Gupta et al, 2001); (3) production of proteases and growth factors providing the environment for cell migration (Betten et al, 2001; Allen et al, 2000; Crawford et al, 2003); and (4) induction of host angiogenesis.

Among the diverse host cellular response related to *H. pylori* associated inflammation or carcinogenesis, some investigators reported that angiogenic growth factors induced by *H. pylori* might be primarily important (Kitadai et al, 2003; Cox et al, 2001; Strowski et al, 2004; Innocenti et al, 2002; Franceschi et al, 2002). Kitadai and colleagues and Cox and colleagues (Cox et al, 2001) 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 colleagues (Strowski et al, 2004) 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 last decade has seen standardisation of the treatment regimens for *H. pylori* eradication, with the use of triple therapy consisting of a PPI 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 (Iwahi et al, 1991; Nakao et al, 1998; Hirai et al, 1995; Tsuchiya et al, 1995; McGowan et al, 1994; Mauch et al, 1993).

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 (Yeo et al, 2004). As Stowski and colleagues (Hirai et al, 1995) reported that *H. pylori* stimulated host VEGF gene expression via the MAPK pathway, we hypothesized 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-1α), and VEGF and, remarkably, angiogenesis induced by *H. pylori* was attenuated by PPI treatment.
II. MATERIALS AND METHODS

A. Cell culture, bacteria strain, and reagents

Human gastric cancer cell lines (AGS, Kato III, SNU-1, SNU-601, MKN-28, and MKN-45) were cultured in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) containing 10% fetal bovine and 100 units/ml penicillin in a humidified 5% CO$_2$ atmosphere. As counter cells of these cancer cells, we cultured normal rat gastric mucosal RGM-1 cells and normal rat intestinal epithelial IEC-6 cells, which were maintained with DMEM-F12 and DMEM, high glucose (Life Technologies, Inc.) supplemented with 10% bovine insulin, respectively, and COS-1 cell, normal human fibroblast cells with RPMI 1640. Human umbilical vein endothelial cells (HUVEC) were obtained by collagenase treatment of umbilical cord veins, as previously described (Jappe EA et al, 1973). Cells were cultured on gelatin coated dishes and propagated in RPMI 1640 medium supplemented with 20% bovine calf serum, 90 ug/ml heparin (Sigma Chemical Co, St Louis, Missouri, USA), and 50 ug/ml endothelial cell growth factor.

A cagA$^+$ and vacA$^+$ standard strain of \textit{H. pylori} (ATCC 43504) was obtained from the American Type Culture Collection (ATCC, Manassas, Virginia, USA). \textit{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% O$_2$, 10% CO$_2$) generated with campy pouch (Becton Dickinson Microbiology Systems, Sparks, Maryland, USA). For inoculation of the bacteria, \textit{H-pylori} were resuspended in phosphate buffered saline (PBS) to an A$_{450}$ of 1.2 units, which corresponds to a bacterial concentration of 5 x 10$^8$ colony forming units.
units (CFU)/ml, and cocultured with AGS cells at a concentration of 5 x 10^7 CFU/ml.

Solutions of pantoprazole (PPZ) and omeprazole were obtained from Altana Pharma AG (Konstanz, Germany) and AstraZeneca, respectively, and lansoprazole (Takeda, Japan) was solved in PBS (adjusted pH 2.0) with HCl overnight at room temperature. PD98059 (50\mu M, extracellular signal regulated kinase (ERK) 1/2 inhibitor; Cell Signaling Technology, Beverly, Massachusetts, USA), SB203580 (10\mu M, p38 inhibitor; Cell Signaling Technology), 1-pyrrolidinecarbodithioic acid (PDTC 100\mu M, ammonium salt, nuclear factor \kappa B (NF-\kappa B) inhibitor; Calbiochem, La Jolla, California, USA), and BAYII-7082 (5\mu M, NF-\kappa 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 \textit{H.pylori}.

B. Tissue samples

Biopsied samples were obtained from five patients (mean age 48 years) with functional dyspepsia without \textit{H.pylori} infection, 20 patients (means age 55 years) with chronic active \textit{H.pylori} positive gastritis, and 18 patients (means age 54 years) with \textit{H.pylori} negative gastritis induced mostly by non-steroidal anti-inflammatory drugs or other cause during gastroscopy. The presence of \textit{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 \textit{H.pylori} negative and if more than two of these tests were positive, the case were defined as \textit{H.pylori} positive. Gastritis was evaluated histologically and scored according to a modified Sydney
classification (Dixon MF et al, 1996); 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.

C. Measurements of Intracellular pH.

Intracellular pH was measured in the monolayers using the pH-sensitive fluorescent probe 2',7'-bis-(2-carboxyethyl)-5-carboxyfluorescein (BCECF). Cells were loaded with BCECF for 10 minutes at room temperature in solution A containing 2.5µM/L BCECF-AM and mounted in the miniature Ussing chamber described for [Ca²⁺]ᵢ measurements. BCFCF fluorescence was recorded and calibrated using a protocol described previously (Namkung W et al, 2003). Briefly, the fluorescence at excitation wavelengths of 490 and 440nm was recorded using a recording setup (Delta Ram; PTI Inc., St Louis, MO), and the 490:440 ratios were calibrated intracellularly by perfusing the cells with solutions containing 145mmol/L KCl, 10mmol/L HEPES, and 5µmol/L nigericin with the pH adjusted to 6.2 to 7.6.

D. Detection of Apoptosis.

Induction of apoptosis was determined by assaying a genomic DNA fragmentation. Briefly, cells were lysed for 15 minutes in 10 mmol/L Tris.Cl (pH 7.4), 5 mmol/L EDTA, and 1% Triton X-100 and centrifuged at 12,000 rpm for 15 minutes. The supernatant was incubated with 0.1mg/ml proteinase K at 37°C for 1 hour and extracted with an equal volume of phenol-chloroform, and the cellular DNA was precipitated with 1:10 volumes of
0.3 mol/L sodium acetate and 2 volumes of absolute EtOH overnight at -70°C. The precipitate was dissolved in 20 µL TE buffer containing 200 µg/ml RNase and incubated for 1 hour at 37°C. The extracted DNA was resolved on 1.8% agarose gel and stained with ethidium bromide.

Caspase-3 and poly(ADP-ribose) polymerase (PARP) proteolysis were assessed by immunoblotting with specific antibodies (Cell Signaling Technology, Beverly, MA). Terminal deoxynucleotidyl transferase (Tdt)-mediated nick end labeling (TUNEL) and annexin V/propidium iodide staining were detected by Fluorescent FragEL DNA fragment detection kit (Oncogene, Boston, MA) and annexin V-FITC apoptosis detection kit (BD Bioscience, San Diego, CA), according to the manufacturer’s instructions, respectively.

E. Western Blot Analysis.

Human gastric cancer cell line, MKN-45 and normal gastric mucosal cell line, RGM-1 cells were incubated with PPZ(0.5 mmol/L PPZ for 0.5, 2, 4 or 8 hours and 0.3 or 0.6 mmol/L PPZ for 24 hours). And Human gastric cancer cell line, AGS cells were incubated with 0, 100, 200, 400 µmmol/L PPZ for eight hours, washed with PBS three times, and then inoculated with *H.pylori* (5 x 10^7 CFU/ml) for 15 minutes (western blotting for ERK1/2) or two hours (western blotting for NFκB). Cells were resuspended in lysis buffer (20mM Tris, pH 7.5, 150mM 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 15,000g for 30 minutes. For documenting NFκB 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 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 Phamacia Biotech, San Francisco, California, USA). Membranes were blocked in 5% non-dry milk and probed with 1:1000 dilution of specific antibodies corresponding to phospho-p38 (Cell Signaling Technology, Beverly, MA), phosphor-ERK, phosphor-JNK, HSP70, HSP60, total ERK, NFkB p65, HSP27 or α-tubulin; all antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA).

F. 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 2 µg 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 Tag kit (Takara, Chiba, Japan) with specific primers as follows: 5'-CTC AAA GTC GGA CAG CCT CA-3' and 5'-CCC CGC AGT TTT CTG CT-3' for HIF-1α; 5'-TCG GCC CGC TCT CGA AAC CAT G-3' and 5'-GGT TCC CGA AAC CCT GAG G-3' for VEGF; 5'-TTG TTG CCA TCA A TG ACC CC-3' and 5'-TGA CAA AGT GGT CGT TGA GG-3' for GAPDH. The PCR reaction was carried out for 28 thermal cycles of 94°C for one minute at 55°C (for HIF-1α 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.
G. 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 manufacture’s instructions (HyCult Biotechnology, Uden, the Netherlands). AGS cells were grown in six well cell culture dishes, incubated in the presence or absence of PPZ for eight hours, and after washing with PBS, cocultured with H.pylori for various times. Culture supernatant (200µl) was used for analysis of IL-8 and VEGF production.

H. Immunohistochemistry for counting vessels in the gastric mucosa.

Immunohistochemistry staining of CD34+ endothelial cells was performed to analyse the degree of angiogenesis in the gastric mucosa of gastric 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 manufacture’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 (x100 magnified field) and presented as mean (SEM) of 20 H.pylori positive and 18 H.pylori negative cases.
I. Immunofluorescence Staining.

Dispersed single cells (2 x 10^5 cells per well) were grown on 22 x 22 x 1 mm glass coverslips in 6-well culture plates. After 24-hour culture, cells were fixed in ice-cold methanol for 5 minutes in a -20°C freezer and permeabilized with 1% Triton X-100/PBS for 10 minutes at room temperature. The cells were blocked with 5% bovine serum albumin for 30 minutes and probed with anti-α subunit of H^+/K^+-ATPase antibodies (1:100 diluted in 5% bovine serum albumin, Santa Cruz Biotechnology) for 2 hours. Cy3-conjugated secondary antibodies were used to visualize under a confocal microscope (BX50F, Olympus, Japan).

J. In vitro angiogenesis assay

In vitro angiogenesis assay was slightly modified from Kitadal and colleagues. Briefly, AGS cells (1 x 10^7 cells/100 mm culture dish) were incubated with 0, 100, 200, or 400 μM PPZ for eight hours, washed with PBS three times, and then inoculated with *H. pylori* (5 x 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 0.4μM 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 dilution in 5% bovine serum albumin; Santa Cruz Biotechnology) for two hours. Cy3-conjugated secondary antibodies were used to visualize under a inverted fluorescence microscope (Olympus, IX71, Tokyo, Japan).

K. Tumor Xenograft.

Subconfluent MKN-45 cells were dissociated with 0.25% trypsin and 1 mmol/L EDTA (Life Technology, Inc.) and suspended in PBS at density of 5 x 10^7 cells/ml. Each mouse was s.c. inoculated with MKN-45 cells (5 x 10^6 per site) on the left and right side of the back on day 0. The animals were randomly divided into two groups (9 per group). Group A received an intratumoral injection of PBS daily from day 14; Group B daily received an intratumoral injection of 0.4 mg/kg PPZ daily from day 14. The shortest and longest intervals, and the volume of each tumor (\( \text{cm}^3 \)) was calculated. Mice were sacrificed at day 22, and isolated tumor tissues were analyzed for microscopic gross finding and TUNEL stain. These studies were approved by the Institutional Animal Care and Use Committee and complied with the highest international criteria for human use of animals in research.

L. Statistics.

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

Human gastric cancer cells were more tolerant of acidity in the culture media than normal cells.

To determine whether cancer cells tolerate acidic conditions better than noncancer cells, we cultured several gastric cancer cell lines (AGS, KATO III, MKN-28, MKN-45, SNU-1, and SNU-601) and noncancer cell lines (RGM-1, IEC-6, and COS-1) in media maintained at different pHs, including 7.4, 6.9, 6.4, 5.9, and 5.4. The cancer cells adapted well to lower pH, whereas the normal cells were much less tolerant of acidity in the culture media (Fig. 1A). At pH 5.4, most of cancer cell lines tested showed >80% viability, but normal cell lines RGM-1 significantly decreased viability by 21.6%.

To investigate whether this difference in cell survival at lower pH was due to ability to dispose or disperse H⁺, we immunocytochemically stained two cancer cells (AGS and MKN-45) and two noncancer cells (RGM-1 and IEC-6) with antibodies against the gastric proton pump, α subunit of H⁺/K⁺-ATPase. We found that there was a much larger amount of proton pump in cancer than noncancer cells (Fig. 1B). Immunofluorescence images showed a predominant expression of the α subunit of H⁺/K⁺-ATPase in the membrane and cytoplasm of cancer cells. These results indicated the enhanced ability to dispose of H⁺ due to overexpression of H⁺/K⁺-ATPase, which might contribute to cancer cell survival in an acidic microenvironment.
Fig. 1. Effects of pH of culture medium on cell viability and cellular expression of H⁺/K⁺-ATPase.

Human gastric cancer cell lines (MKN-45, MKN-28, AGS, Kato III, SNU-601, and SNU-1) and noncancer cell lines (RGM-1, Cos-1, and IEC-6) were cultured in media at different pH for 24 hours, and cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (A). Expression of H⁺/K⁺-ATPase, α-subunit was detected with specific antibodies and visualized with Cy3-conjugated secondary antibodies. Note that the α-subunit of H⁺/K⁺-ATPase was more highly expressed in the plasma membrane and cytoplasm of cancer than noncancer cells (B).
Treatment of PPZ significantly inhibits cancer cell viability in a pH-dependent manner.

Because PPZ is a protonatable weak base, which can convert to active form in an acidic environment with a low pH, we tested the effects of PPZ on cell growth in culture media maintained at various pHs (Fig. 2). Nevertheless, cancer cells showed tolerance of acidity in culture media as shown in Fig. 1A, and administration of the proton pump inhibitor PPZ led to significant reduction of cancer cell survival (Fig. 2A). At pH 5.0, PPZ treatment reduced cell viability by 6.9% on average in MKN-45 cancer cells (Fig. 2A). On the contrary, normal cell line RGM-1 did not showed a significant difference in cell viability between the PPZ-treated and nontreated groups (data not shown). Despite resistance of cancer cells to the acidic environment, cancer cells were much more susceptible to growth inhibition of PPZ at a lower pH.

We evaluated whether this attenuation of cancer cell viability is induced by a decrease of intracellular pH due to the blocking of disposal of intracellular H⁺ by specific protonation of this drug. As shown in Fig. 2B, PPZ treatment significantly increased acidity of intracellular pH in AGS, MKN-28, and MKN-45, but SNU-601 did not show a remarkable change (Fig. 2B). Of note, AGS decreased intracellular pH from 7.6 to 7.2. These findings showed that PPZ-induced cell death was caused by the specific protonation of this proton pump inhibitor.

We also investigated whether other proton pump inhibitors such as omeprazole and lansoprazole has a similar effect to pantoprazole on cancer cell viability. Human gastric MKN-45 cells were cultured with each proton pump inhibitor in pH 6.0 of media for 24 hours, and cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-
diphenyltetrazolium bromide assay. The results showed that whereas the PPZ or omeprazole-treated cells remarkably reduced cell viability in a dose-dependent manner, lansoprazole did not show any changes in cancer cell viability (Fig. 3). This difference suggested that the decreased cell viability comes from the specific effect of proton pump inhibition. Omeprazole and PPZ are available for parenteral injection, but lansoprazole is only available for oral administration, suggesting that lansoprazole lost its proton pump inhibiting ability in aqueous form.
In different pH media (7.4, 6.9, 6.4, 5.9, 5.0), gastric MKN-45 cancer cells were incubated in the presence or absence of 0.5 mmol/L PPZ, and cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (A). Although MKN-45 showed resistance to acidity of culture media, PPZ significantly decreased this cancer cell viability in a pH-dependent manner. To evaluate effect of PPZ on intracellular pH ($pHi$) of cancer cell, gastric cancer cells were plated in a 6-well culture dish, treated with 0.5 mmol/L PPZ for 16 hours, and measured according to Materials and Methods as described previously (B).
To compare effect of other proton pump inhibitors (omeprazole and lansoprazole) with PPZ on cancer cell viability, MKN-45 cells were cultured in the presence of each proton pump inhibitor indicated at pH 6.0 for 24 hours and were measured cell viability by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Cell viability (%) was calculated by formula as follows: Cell viability (%) = (sample absorbance - total absorbance)/(spontaneous absorbance - total absorbance) × 100. Total absorbance was obtained from absorbance of 0.1% Triton X-100-treated cells as negative control, and spontaneous absorbance indicated absorbance of PPZ-untreated control cells.

Fig. 3. Effect of proton pump inhibitors on gastric cancer cell viability.
**PPZ selectively induced apoptosis cell death in human gastric cancer cells.**

To determine whether PPZ preferentially induces apoptosis in cancer cells, we administered this agent to two cell lines, a normal gastric mucosal cell line, RGM-1, and a gastric cancer cell line, MKN-45 (Fig. 4). Although normal gastric mucosal RGM-1 cells significantly reduced cell viability in a low pH media as shown in Fig. 1A, an apoptotic genomic DNA fragmentation was detected by neither pH change nor PPZ treatment (Fig. 4A). However, human gastric cancer cell MKN-45 showed a significant apoptotic genomic DNA fragmentation, caspase-3 and its substrate, PARP, were cleaved by PPZ treatment in a dose-dependent manner in gastric cancer cells but not detected in normal gastric mucosal cell (Fig. 4B). PPZ also induced alteration of phosphatidylserine distribution and permeability of plasma membrane only in cancer that which were detected by annexin V/propidium iodide staining (Fig. 4C). These results indicated much higher vulnerability and selective sensitivity to apoptosis in gastric cancer MKN-45 cells than normal mucosal RGM-1 cells.
Fig. 4. Selective induction of apoptosis with PPZ.

Cells were cultured in the presence or absence of PPZ for 24 hours, and induction of apoptosis was detected by genomic DNA fragmentation (A), caspase-3 activation and PARP cleavage (B), and localization of annexin V and propidium iodide (C). Annexin V-positive cells were stained green, and red represented propidium iodide-positive cells.
PPZ suppressed ERK phosphorylation in human gastric cancer cells.

To assay the involvement of MAPKs in PPZ-induced apoptosis, phosphorylation of ERKs, JNKs/stress-activated kinases, and p38 after PPZ treatment was detected (Fig. 5A and B). In MKN-45 cells, PPZ significantly and selectively inhibited the phosphorylation of ERK and increased the phosphorylation of p38 but had no apparent effects on the phosphorylation of JNK. After 8-hours treatment of 0.5 mmolL PPZ, ERK phosphorylation was completely inhibited in MKN-45 cells despite an equal amount of total proteins controlled by α-tubulin (Fig. 5A). In addition, inhibition of p38 by SB203580 blocked PPZ-induced apoptosis in MKN-45 cells (data not shown). As shown in Fig. 5B, RGM-1 cells did not change the degree of phosphorylation of ERK and p38, but phosphorylation of JNK was significantly decreased in a time-dependent manner. Taken together, these findings suggest that inhibition of ERK phosphorylation may be responsible for the attenuation of cancer cell survival, whereas activation of p38 contributes to proton pump inhibitor-induced apoptosis.
Fig.  5.  Distinct MAPK signaling in cancer cells (A) and noncancer cells (B) by PPZ.

Cells were treated in the presence or absence of 0.5 mmol/L PPZ for various times, and total proteins were extracted from the cells, electrophoresed on 12% SDS-PAGE gels, and transferred to polyvinylidene difluoride membranes. The membranes were probed with specific antibodies for p-ERK, p-P38, p-JNK, and α-tubulin, respectively.
Overexpression of HSP27 and HSP70 played a cytoprotective role in PPZ-induced apoptosis of normal gastric mucosal cells.

Interestingly, we found that HSP70 and HSP27 were overexpression in RGM-1 after PPZ treatment but not in MKN-45 cells (Fig. 6A and B). Notably, treatment of 0.6 mmol/L PPZ in RGM-1 cells showed a 3.3- and 36.3-fold increased of HSP27 and HSP70 compared with that of nontreated cells, respectively (Fig. 6C). However, HSP27 and HSP70, as well as HSP60, were not grossly changed in PPZ-treated gastric cancer MKN-45 cells (Fig. 6A). To compare the HSP induction, we also treated geranylgeranylacetone (GGA), which is well known as a HSP inducer in gastric mucosal cells. GGA slightly increased HSP60 and HSP27 in MKN-45 cell, but deceased HSP60 and HSP27 in RGM-1. In comparison with GGA, the ability of PPZ to induce HSP70 is much higher at 5.6-fold than GGA. These data suggest that overexpression of HSP27 and HSP70 might play a cytoprotective role in PPZ-induced apoptosis of normal gastric mucosal cells.
Fig. 6. Effect of PPZ on expression of HSP70 and HSP27 in gastric cancer and noncancer cells.

RGM-1 (A) and MKN-45 cells (B) were treated with PPZ or generylgenerylacetone (GGA) for 24 hours, and expression of HSP70, HSP60, and HSP27 was assayed by immunoblotting with specific antibodies. Note that PPZ induced expression of HSP70 and HSP27 in RGM-1 normal gastric mucosal cells but not in MKN-45 gastric cancer cells. Relative intensities of HSP70 and HSP27 compared with nontreated control cells were represented in C.
In a xenograft model of nude mice, administration of PPZ significantly inhibited tumorigenesis and induced large-scale apoptosis of tumor cells.

We also evaluated the antitumorigenic effects of PPZ in a gastric cancer xenograft model (Fig. 7). The animals were randomly divided into two groups: an intratumoral injection of PBS and an intratumoral injection of 0.4 mg/kg PPZ. Intratumor administration of PPZ significantly suppressed tumor growth in athymic nude mice, with decreased in tumor volume at day 22 of 44.69% compared with mice injected with PBS (Fig. 7A and B). The isolated tumor from mice with intratumor administration of PPZ was remarkably smaller than that of mice intramorally injected with PBS (Fig. 7C). Histopathological examination revealed that PPZ treatment provoked considerable apoptosis. Only a few tumor cells survived, and most tumor cells were replaced by apoptosis cells (Fig. 7D). TUNEL staining also showed considerably higher apoptotic cell death in the PPZ-treated group compared with the control group (Fig. 7E).
Fig. 7. Antitumorogenic potency of PPZ in xenograft model.

MKN-45 human gastric cancer cells were inoculated s.c. into the backs of athymic nude mice. PPZ (40mg/kg) was injected intratumorally (IT) 14 days after xenograft. Mice were sacrificed 22 days after tumor inoculation. Tumor volume was measured and represented (A and B). The isolated tumors (C) were sectioned and processed for H&E staining (D, ×100 and ×400 magnification) and TUNEL assay (E, ×100 and ×200 magnification); bars, ±SD.
Distinct expression of CD34⁺ blood vessels between *H. pylori* positive and negative gastric 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, 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. 8A (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. 8A (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. 8B. While *H. pylori* negative gastritis samples had a mean of 7.2 (SEM 0.8) blood vessels per x100 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. 8A (b, d), arrow) were thicker and larger than those of *H. pylori* negative cases (Fig. 8A (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 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. 9). 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. 9B).
Fig. 8. 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; c, ×200) and *H. pylori* positive (b, ×100; d, ×200) 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 color (arrow), counterstained with haematoxylin showing a blue color. (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.
Hp, *Helicobacter pylori*.

Fig. 9. Expression of hypoxia inducible factor 1 (HIF-1α) mRNA in human gastric mucosa.

(A) Reverse transcription–polymerase chain reaction of HIF–1α 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–1α mRNA is represented as mean intensity/mm². HIF–1α 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).
Suppression of H. pylori induced in vitro angiogenesis by gastric PPZ

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. 10B) compared with conditioned media of non-H. pylori infected AGS (Fig. 10A). CD31 immunofluorescence staining showed a dense intensity of CD31 molecules in HUVEC cells incubated with the culture supernatants of H. pylori infected AGS (Fig. 10C) 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. 10A, and 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 PPZ (200µM or 400µM, for eight hours) on AGS cells prior to H. pylori inoculation significantly inhibited tubular formation (Fig. 10D, and E) and CD31 expression of endothelial HUVEC (Fig. 10I, and J). However, no significant changes were noted in HUVEC incubated with 400µM PPZ alone (Fig. 10C, and H), suggesting that PPZ itself did not influence tubular formation of HUVEC. The data clearly indicate that PPZ suppressed H. pylori induced in vitro angiogenesis, suggesting that antiangiogenic treatment with PPZ could be a promising therapeutic approach for H. pylori associated carcinogenesis.
AGS cells (1×10⁷ cells/100mm² culture dish) were incubated with 0, 200, or 400 µM proton pump inhibitor (PPZ) for eight hours, washed with phosphate buffered saline three time, and inoculated with *Helicobacter pylori* (5×10⁷ 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 µM pore filter to remove *H. pylori* and then added to the HUVEC culture which was change 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

**Fig. 10. In vitro angiogenesis assay.**
incubated with conditioned media of *H. pylori* infected AGS; (C, H) HUVEC cells incubated with conditioned media of 400 µM PPZ treated AGS; (D, I) HUVEC cells incubated with conditioned media of 200 µM PPZ/*H. pylori* infected AGS; (E, J) HUVEC cells incubated with conditioned media of 400 µM PPZ/*H. pylori* infected AGS.
Production of proangiogenic factors from H. pylori infected gastric epithelium and its inhibition by PPZ

Following *H. pylori* infection, AGS cells significantly secreted VEGF and IL-8, well characterized as proangiogenic factors, in a time dependent manner (Fig. 11A). 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. 11A). We also examined mRNA expression of these angiogenic factors using RT-PCR analysis (Fig. 11B). 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. 11B). IL-8 mRNA was also significantly induced after *H. pylori* infection (Fig. 11B). All of these results suggest that synthesis of angiogenic epithelial cells, which could induce proliferation and differentiation of endothelial cells.

Because PPZ showed a strong antiangiogenic action in the in vitro angiogenesis assay (Fig. 10), we measured the effect of PPZ on expression of these angiogenic factors (Fig. 12). Secretion of IL-8 in the supernatants of *H. pylori* infected AGS cells was found to be remarkably suppressed after PPZ treatment in a dose dependent manner (Fig. 12A). 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 PPZ pretreatment. Pretreatment with PPZ showed a considerable regulatory effect on *H. pylori* mediated VEGF synthesis (Fig. 12A). Suppression of these angiogenic factors by PPZ was evidenced by transcriptional inhibition of the genes (Fig. 12B). At 400 µM of PPZ, expression of VEGF and HIF-1α seemed to decline relevant to that of control AGS cells.
Fig. 11. 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.
Fig. 12. Effects of proton pump inhibitor (PPI) on expression of angiogenic growth factors interleukin 8 (IL-8), hypoxia inducible factor 1 (HIF-1α), and vascular endothelial growth factor (VEGF).

(A) To examine the inhibitory effect of PPI on *Helicobacter pylori* induced angiogenic growth factor expression, AGS cells (1×10⁷ cells/100 mm² culture dish) were incubated with 0, 50, 100, 200, or 400 µM PPI for eight hours, washed with phosphate buffered saline three times, and inoculated with *H. pylori* (5×10⁷ CFU/ml) for 16 hours. Production of IL–8 (top) and VEGF (bottom) was measured in culture supernatant of the cells by ELISA. (B) Total RNA extracted from cells was used in the reverse transcription–polymerase chain reaction analysis of HIF-1α and VEGF.
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 NFκB on H. pylori induced angiogenesis using specific inhibitors (Fig. 13). PD098059 (50 μM), one of the ERK inhibitors, strongly inhibited H. pylori induced HIF-1α and VEGF expression, and SB203580 (10 μM), a p38 inhibitor, was also able to inhibit expression of these angiogenic factors. PDTC (ammonium salt, 100 μM) a NFκB inhibitor, potently suppressed HIF-1α and VEGF expression induced by H. pylori. BAY11-7082 (5 μM) 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 NFκB pathway.
Fig. 13. Involvement of mitogen activated protein kinase and nuclear factor κB (NFκB) in *Helicobacter pylori* induced mRNA expression of hypoxia inducible factor 1 (HIF-1α) and vascular endothelial growth factor (VEGF).

Prior to inoculation with *H. pylori*, AGS cells were treated with each inhibitor (50 μM PD08059, 10 μM SB203580, 100 μM PDTC, or 5 μM BAYII-7082 (BAY)) for eight hours, and their effects on *H. pylori* induced HIF-1α 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-pyrrolidinecarbodithioic acid, ammonium salt, NFκB inhibitor; BAY, BAYII-7082, NFκB inhibitor.
PPZ disturbs H. pylori induced signaling 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. 13) and that the anticancer action of PPZ is fundamentally attributable to inhibition of phosphorylation of MAPK ERK1/2, we evaluated whether the antiangiogenic activity of PPZ is caused by block of ERK activation. Western blot analysis with phosphor-ERK antibodies was performed to determine the influence of PPZ on MAPK ERK1/2 activation related to H. pylori induced angiogenesis (Fig. 14A, and B). The ERK inhibitor, PD098059, and the p38 inhibitor, SB203580, decreased phosphorylation of ERK1/2. Interestingly, PPZ completely attenuated phosphorylation of ERK1/2 inhibitor PD098059 (Fig. 14A). These inhibitory actions of PPZ against ERK phosphorylation were dose dependent (Fig. 14B) and maximal inhibitory activity was seen after four hours (data not shown). However, PPZ did not have any effect on nuclear translocation of NFκB, suggesting that the antiangiogenic effect of PPZ seems to be independent of suppression of NFκB (Fig. 14C). In summary, angiogenesis induced by H. pylori was attenuated by PPZ treatment, which was caused by inactivation of the MAPK pathway, one of principal signals for H. pylori induced angiogenesis.
Fig. 14. Deactivation of mitogen activated protein kinase (MAPK) extracellular signal regulated kinase (ERK)1/2 signaling 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 phosphor-ERK antibodies. (B) Effect on phosphorylation of ERK at different concentration of PPI. (C) Effect of PPI on H.pylori induced nuclear factor κB (NFκB) translation 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. Nucleic proteins were isolated from cells and subjected to western blotting using specific NFκB in nuclear fractions compared with total NFκB. PD, PD098059 (ERK1/2 inhibitor), SB, SB203580 (p38 inhibitor).
IV. DISCUSSION

Because the ultimate aim of anticancer treatment is to kill only the cancer cells, the therapeutic efficiency of anticancer agents could be increased by specificity and selectivity to cancer cells. Current understanding of anticancer strategy has led to attempts to screen for agents that selectively increase in cancer cells or to use apoptosis pathways specific for tumor cells. Therefore, our current findings suggested that the gastric proton pump inhibitor PPZ has very considerable advantages as anticancer treatment based on the following novel findings. First, PPZ selectively induced apoptosis in cancer cells, and this PPZ-induced apoptosis may be caused by suppressing ERK phosphorylation. This H+/K+^-ATPase inhibitor simultaneously stimulated phosphorylation of p38 as well as deactivation of ERK in a dose- and time-dependent manner only in cancer cells. The different regulation of the individual MAPK subfamily by the proton pump inhibitor seems to be better considered as anticancer agents with high selectivity to cancer, because generally p38 is involved on apoptosis and stress signaling pathway, whereas ERK is activated by stimuli of cell growth and differentiation. Second, noncancer cells have mechanisms to counteract the PPZ-induced apoptosis by the induction of antiapoptotic molecules HSP70 and HSP27. We observed that PPZ-induced significant amounts of HSP70 much more than GGA, an agent generally known to induce HSP70 as a key action. Thus, the H+/K+^-ATPase inhibitor may act to selectively induce apoptotic cell death in cancer cells without having any significantly adverse effects on the surrounding noncancer cells, and the feature of this agent contributes to safety in an aspect of clinical application. Third, PPZ would be selectively conversion of
the active form under the hypoxic and acidic condition, resulting in induction of significant apoptosis. Just like in the canaliculi of parietal cell, pH of inner space of the tumor was reported to be below 6.8 (Frenzel et al, 1994; Gillies et al, 1994). The finding shown in Fig. 2 showed that with lower pH of media, higher activities of apoptosis were observed. In all, these results suggest that PPZ can be used clinically as an anticancer agent.

One of the well-known properties of cancer cells is aerobic glycolysis, which may cause tumor acidification (Warburg et al, 1930; Holm et al, 1995; Vaupel et al, 1989). The cancer cell will take in glucose and form lactic acid, which will largely dissociate into the lactate ion and proton (H⁺). The lactate ion and H⁺ produced from the glycolysis are effluxed into the extracellular fluid leading to low extracellular pH and high intracellular pH of cancer. There are some mechanisms involved in the regulation of tumor pH; the main mechanism by which proton (H⁺) is exported is by the sodium-hydrogen antiport, using the energy of the Na⁺ gradient (Karamazyn et al, 1999; Wakabayashi et al, 1997). Tumor cells may possess an additional mechanism for H⁺ export via a vacuolar H⁺-ATPase (V-ATPase) of plasma membrane, the bicarbonate transporter, and the proton-lactate synporter (Martinez-Z et al, 1993; Finbow et al, 1997). In the present study, we used the H⁺/K⁺-ATPase inhibitor for blocking the H⁺ export of tumor cells. The H⁺/K⁺-ATPase inhibitor successfully suppressed tumor cell viability by inducing apoptotic cell death. Thus, these findings implicate that blockage of another kind of proton pump predominantly expressed in tumor cells could be used as a promising anticancer drug.

Several inhibitors have been found to interact with vacuolar-type H⁺-ATPase, interfering with both ATP hydrolysis and proton translocation activities (Bowman et al,
1988; Zhang et al, 1992; Dross et al, 1993). Theses inhibitors can divided largely into two classes: inhibitors acting at a soluble cytoplasmic domain (N-ethylmaleimide and 4-nitrobenzo-2-oxa-1,3-diazole chloride) and inhibitors acting at transmembrane sites (dicyclohexyl-carbodimide, Bafilomycina A1, and Concanamycin A). Several research groups have already reported that these inhibitors of vacuolar-type H⁺-ATPase can induce apoptotic cell death in several human cancer cell lines including pancreatic cancer, hepatocellular carcinoma, and B-cell hybridoma cells (Nishihara et al, 1995; Ishisaki et al, 1999; Ohta et al, 1998; Aiko et al, 2002; Hasimoto et al, 2002). They also proved the involvement of cytochrome C release and caspase activation in vacuolar-type H⁺-ATPase inhibitor-induced apoptosis. The specific inhibitors of mammalian vacuolar-type H⁺-ATPase belonging to the benzolactone enamide class, such as salicylihalamide, lobatamides, and oximidines, were developed and appear promising as anticancer agents (Erickson et al, 1997; Boyd et al, 2001). However, some reports are contradicting the proapoptotic effect of the proton pump inhibitor, suggesting that the inhibitor of mitochondria F₀F₁-ATPase proton pump, oligomycin, prevented apoptotic cell death (Schwerdt ea al, 2003; Matsuyama et al, 1998; Shchepina et al, 2002).

However, there remain two limitations for anticancer application of a vacuolar-type H⁺-ATPase inhibitor like concanamycin A or bafilomycin A. The first one is nonselectivity of apoptosis of vacuolar-type H⁺-ATPase inhibitor, and the second problem is that the vacuolar-type H⁺-ATPase gene is considered a “housekeeping gene” expressed indiscriminately on every cell (Torigoe et al, 2002). Hence, there might be similar cytotoxicity provoked by current cytotoxic anticancer drugs. The feasibility of clinical
application of these factors is difficult despite strong apoptotic-inducing capability. On the other hand, PPZ showed selective induction of apoptosis in cancer cells. It rendered noncancerous cells escaping from apoptotic activities through the induction of antiapoptotic signaling molecules. Moreover, the fact the proton pump inhibitor is a prodrug requiring protonation after administration brings more hope that protonation is more easily performed within tumor tissues.

In conclusion, our findings provide novel mechanistic insight into the anticancer target of gastric proton pump, H\(^+\)/K\(^+\)-ATPase, and expand the repertoire of clinical use of gastric proton pump inhibitor as an anticancer drug by implicating the selective induction of apoptosis in cancer cells.

After \(H.pylori\) infection, the signal transduction enzymes, MAPK ERK1/2 and NF\(\kappa\)B, are activation and these molecules are responsible for transcriptional activation of angiogenic growth factors, including IL-8, HIF-1\(\alpha\), 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 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 carcinogenesis, and the inhibitory effects of PPIs on \(H.pylori\) induced angiogenesis. PPI treatment efficiently inhibited IL-8, VEGF, and HIF-1\(\alpha\) expression in \(H.pylori\) infected gastric epithelial cells,
which was due to inactivation of MAPK signaling induced by *H. pylori* infection. 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 (Jordan et al, 2004; Blagosklonny et al, 2004). Among the proangiogenic factors known, VEGF response one of the most potent stimuli of neangiogenesis. Although previous studies found that enhanced VEGF gene expression contributed to the healing of peptic lesions in the stomach (Baatar et al, 2002; Jones et al, 2001), here we demonstrated that *H. pylori* infection stimulation of blood vessels in the mucosa layer, which might be positive 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 (Takahashi et al, 1996; Maeda et al, 1999; Kanai et al, 1998). Gastric adenocarcinomas frequently showed high levels of VEGF expression (Takahashi et al, 1996; Maeda et al, 1999), and neutralization of circulating VEGF with specific VEGF antibodies potently reduced the growth of gastric cancer (Kanai et al, 1998). Potent stimulators of angiogenesis related to *H. pylori* infection, *H. pylori* VacA toxin (Caputo et al, 2003), reactive oxygen species synthesized from neutrophils or macrophages (Park et al, 2003), *CagA* pathogenicity
islands (Cox et al, 2001; Innocenti et al, 2002), 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 (Cox et al, 2001; Innocenti et al, 2002).

The PPI PPZ, a substituted 2-pyridyl methyl/sulfinyl benzimidazole derivative, is a prodrug requiring protonation under acidic conditions for functions activation, accumulates selectively in the acidic gastric luminal space, and ultimately inhibits acid secretion by covalent binding with cysteins residues on the α-subunit of H'K'-ATPase. These PPIs have been universally used with antibodies for the eradication of *H.pylori* and several types of acid related diseases, including gastro-oesophageal reflux diseases, peptic ulcer diseases, and Zollinger-Ellison syndrome (Sachs et al, 1997; Fitton et al, 1996). Increased gastric pH by PPIs stimulates resting *H.pylori* to activate metabolically and thus *H.pylori* are more susceptible to antibiotics (Iwahi et al, 1991; Nakao et al, 1998; Hirai et al, 1995; Tsuchiya et al, 1995; McGowan et al, 1994; Mauch et al, 1993). 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 (Yeo et al, 2004). 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 \textit{H. pylori} induced intracellular signaling via inhibition of MAPK activation. Compared with inhibitors of ERK1/2 or p38, PPZ showed stronger inhibitory activities than these (fig. 14), in a dose dependent manner. However, the influence of this drug on NFκB transcriptional activation was minor (fig. 14).

In conclusion, we have shown that considerable angiogenic activities were stimulated in the gastric mucosa after \textit{H. pylori} infection, for which increasing proangiogenic growth factors from gastric epithelial cells were responsible. PPIs could have significant inhibitory activities against \textit{H. pylori} associated angiogenesis. Therefore, the current data indicate that as \textit{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 \textit{H. pylori} provoked angiogenesis.
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위산억제제의 새로운 작용기전: 헬리코박터 파이로리균에 의해 유도되는 신생혈관생성을 억제하고 위암세포에만 선택적으로 세포자사멸을 유도한다.

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암세포는 급격한 세포분열을 위한 대사 작용으로 인해 암세포내에 많은 양의 수소이온이 축적이 되고 세포벽 환경도 배출된 수소이온들로 인해 낮은 pH가 형성되어 있다. 따라서, 대사 작용 결과 생성된 수소이온을 세포밖으로 충분히 퍼낼 수 있는 능력이 요구된다. H⁺/K⁺-ATPase 는 세포내의 H⁺와 세포밖의 K⁺를 교환하는 역할을 하여 위의 산성화에 기여하는 효소이다. 이러한 사실들에 기인하여, 우리는 Gastric H⁺/K⁺-ATPase inhibitor (위산억제제)의 하나인, Pantoprazole (PPZ) 을 사용하여 암세포가 수소이온을 세포벽으로 퍼내는 능력을 차단하면, 암세포의 성장을 억제하거나 암세포를 죽일 수 있을 것이란 가정을 세웠다. 그리고, 헬리코박터 파이로리균의 감염으로 인한 mitogen activated protein kinases (MAPKs)의 활성화
성화가 숙주의 신생혈관생성을 유도하고, 더 나아가 위암화에 기여한다는 것은 많이 알려진 사실이나, 그 과정을 억제하는 방법에 관하여는 밝혀진 것이 많지 않다. 그러므로, 우리는 위암예방제가 MAPK ERK1/2의 인산화를 강하게 억제하는 능력이 있다는 것을 보고하였기에, 헬리코박터 파이로리균 감염에 의한 MAPK ERK1/2의 활성화를 위암예방제로 억제할 수 있고 그 결과 신생혈관생성도 억제할 수 있는가를 조사해 보았다.

PPZ에 의해, 양새포가 세포자사멸에 이르는가를 알아보기 위하여 genomic DNA fragmentation, terminal deoxynucleotidyl transferase (Tdt)-mediated nick end labeling assay, 그리고 annexin V 염색법을 수행하였다. 그리고, Mitogen-activated protein kinase와 Heat shock protein의 발현과 양새포의 세포자사멸과의 상관관계를 알아보기 위하여 특정항체를 이용한 immunoblot을 이용해 검증하였다. 그리고, PPZ가 생체내에서 항암효과가 있는지 검증을 위하여 nude mice를 이용한 Xenograft model 실험을 수행하였다. 그리고, 헬리코박터 파이로리균의 감염과 신생혈관생성과의 관계를 알아보기 위해, 20명의 헬리코박터 파이로리 양성인 환자와 18명의 헬리코박터 파이로리 음성인 환자의 조직절편에서 CD34⁺ 혈관의 상대적 발현량을 확인해 보았다. 그리고, reverse transcription-polymerase chain reaction(RT-PCR)을 이용해 hypoxia inducible factor 1(HIF-1α)와 vascular endothelial growth factor(VEGF)의 발현량을 비교해 보았고, ELISA를 이용해 interleukin 8과 VEGF의 분비도 확인해 보았다. 그리고, in vitro angiogenesis assay를 이용하여, 헬리코박터 파이로리균의 감염이 human umbilical vein endothelial cells(HUVEC)의 혈관모양생성을 직접적으로 유도하는지도 확인해 보았다. 그리고, MAPK와 nuclear factor κB(NFκB)의 활성화도 immunoblotting으로 확인해 보았다.
그 결과로, 암세포에 PPZ를 처리해 주면 세포자사멸에 이르는 것을 확인하였고 이때, extracellular signal-regulated kinase(ERK)의 활성화가 저해되는 것을 확인하였다. 이와 대조적으로, 정상세포에서는 HSP70과 HSP27 같은 항세포자사멸인 자들의 과발현을 통하여 PPZ에 의해 유도되는 세포자사멸에 저항성을 보이는 것을 확인하였다. 그리고, nude mice를 이용한 xenograft model에서도 PPZ의 접종이 암화의 진행을 억제하고, 암세포의 세포자사멸을 유도한다는 것을 확인하였다. 그리고, 헬리코박터 파이로리균 양성인 위암 환자 (40.9±4.4)에서 헬리코박터 파이로리균 음성인 위암 환자 (7.2±0.8) 보다 CD34+ 혈관의 발현량이 현저히 증가되어 있는 것을 관찰하였고, HIF-1α의 발현 상관관계도 같은 결과를 보여주었다. 위상포세포에 헬리코박터 파이로리균을 감염시켜 얻은 배양액을 가지고 HUVEC 세포의 배양액에 혼합하여 배양했을 때, HUVEC 세포가 혈관모양을 생성하는 것을 확인할 수 있었다. 그러나, 여기에 위산억제제를 처리해주면 현저하게 HUVEC 세포가 혈관모양을 생성하는 것을 억제해 주었다. 그리고, 헬리코박터 파이로리균의 감염이 MAPK의 활성화와 전사인자인 NFκB의 활성화를 통해 혈관생성인자인 HIF-1α와 VEGF의 발현을 증가시킨다는 것을 확인할 수 있었다. 이러한 과정에, 위산억제제가 MAPK ERK1/2의 인산화를 효과적으로 억제해 헬리코박터 파이로리균의 감염에 의한 신생혈관생성 과정을 억제하는 효과를 보이는 것을 확인할 수 있었다.

결론적으로, 위산억제제가 생체내와 세포수준에서 위암세포의 세포자사멸을 유도하고, 헬리코박터 파이로리균의 감염으로 인한 신생혈관생성을 억제하는 능력이 있다면, 이 위산억제제가 더 나아가 항암치료에 이용될 수도 있고, 또한, 헬리코박터 파이로리균과 관계된 위암화의 진행을 억제할 수 있는 치료제로의 역
할도 기대해 볼 수 있겠다.

핵심어 : 위산억제제, 세포자사멸, 위암, H’/K’-ATPase, 항암효과, 헬리코박터 프로리균, 신생혈관생성, HIF-1α, VEGF, 암화과정