Research Article

Cancer Prevention Research

Novel Application of Proton Pump Inhibitor for the Prevention of Colitis-Induced Colorectal Carcinogenesis beyond Acid Suppression

Yoon Jae Kim[1], Jeong Sang Lee[2], Kyung Sook Hong[2], Jun Won Chung[1], Ju Hyun Kim[1], and Ki Baik Hahm[1,2]

Abstract

Colitis-associated cancers arise in the setting of chronic inflammation wherein an "inflammation-dysplasia-carcinoma" sequence prevails. Based on our previous findings in which the proton pump inhibitor could impose significant levels of anti-inflammatory, antiangiogenic, and selective apoptosis induction beyond gastric acid suppression, we investigated whether omeprazole could prevent the development of colitis-associated cancer in a mouse model induced by repeated bouts of colitis. Omeprazole, 10 mg/kg, was given i.p. all through the experimental periods for colitis-associated carcinogenesis. Molecular changes regarding inflammation and carcinogenesis were compared between control groups and colitis-associated cancer groups treated with omeprazole in addition to chemopreventive outcome. Nine of 12 (75.0%) mice in the control group developed multiple colorectal tumors, whereas tumors were noted in only 3 of 12 (25.0%) mice treated with daily injections of omeprazole. The cancer-preventive results of omeprazole treatment was based on significant decreases in the levels of nitric oxide, thiobarbituric acid–reactive substance, and interleukin-6 accompanied with attenuated expressions of tumor necrosis factor-α, inducible nitric oxide synthase, and cyclooxygenase-2. The expressions of matrix metalloproteinase (MMP)-9, MMP-11, and MT1-MMMP were significantly decreased in mice treated with omeprazole in accordance with significant decreases in the number of β-catenin–accumulated crypts. A significant induction of apoptosis was observed in tumor tissue treated with omeprazole. Omeprazole could block the trophic effect of gastrin in colon epithelial cells. The significant anti-inflammatory, antioxidative, and antimutagenic activities of omeprazole played a cancer-preventive role against colitis-induced carcinogenesis, and our novel *in vivo* evidence is suggestive of chemopreventive action independent of gastric acid suppression. *Cancer Prev Res; 3(8); 963–74. ©2010 AACR.*

Introduction

Ulcerative colitis (UC) is a form of chronic inflammatory bowel disease (IBD) that usually takes a clinical course of repeated exacerbation and remission, but less commonly presents with an unremitting, fulminant course (1, 2). Another feature of UC is an increased risk for the development of colitis-associated cancer, also known as "colitic cancer" (3, 4). Although both sporadic colorectal cancer and colitic cancer arise from dysplastic precursor lesions and share some molecular alterations, the nature of the dysplasia and the frequency and timing of several of the key molecular changes differ enough to support our hypothesis that in contrast to sporadic colorectal cancer, colitic cancer could be prevented with either the early intervention of effective anti-inflammatory strategies or through cancer surveillance using biomarkers (5, 6). With regard to biomarkers for colitic cancer risk, aneuploidy, p53, mucin-associated *sialyl*-Tn antigen expression, and loss of transgelin hold promise (7–9); however, they are not yet practical for clinical use because a panel of genes or proteins engaged in colitic cancer needs further validation. Therefore, as a potential biomarker for tumor surveillance, it is important to investigate whether a potent anti-inflammatory drug or strategy could be applied for the prevention of colitic cancer through either the abolishment or the delay of carcinogenic processes initiated or promoted by sustaining inflammation.

Proton pump inhibitors (PPI) have been shown to exert anti-inflammatory and cytoprotective actions independent of gastric acid inhibition (10–13). Even though their fundamental pharmacologic actions are considered to be due to their gastric antisecretory actions, which results from a nearly complete inhibition by irreversibly

Authors' Affiliations: [1]Department of Gastroenterology, Gachon Graduate School of Medicine and [2]Laboratory of Translational Medicine, Gachon University Lee Gil Ya Cancer and Diabetes Institute, Gachon University of Medicine and Science, Incheon, Korea

Corresponding Author: Ki-Baik Hahm, Laboratory of Translational Medicine, Lee Gil Ya Cancer and Diabetes Institute and Department of Gastroenterology, Gachon Graduate School of Medicine, Gachon University Gil Hospital, 7-45 Songdo-dong, Yeonsu-gu, Incheon 406-840, Korea. Phone: 82-32-899-6055; Fax: 82-32-899-6054; E-mail: hahmkb@hotmail.com or hahmkb@gachon.ac.kr.

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inhibiting gastric H^+/K^+-ATPase in parietal cells, they could mediate either anti-inflammatory effects through the induction of heme oxidase-1 (11), inhibition of angiogenic growth factors (12), oxidation of Kelch-like ECH-associated protein 1 (10), or selective induction of apoptosis in cancer cells (13). All of these additional actions of PPI suppression suggest the possibility of new therapeutic avenues for the treatment of inflammatory disorders and the prevention of inflammation-associated carcinogenesis.

Even though omeprazole is a PPI specifically targeted for blocking hydronium efflux by inhibiting proton pumps in the apical portion of parietal cells, we and other investigators (10–13) have suggested that they could impose selective cancer cell apoptosis, antioxidative action as shown by NF-E2–related factor 2 (*nrf*-2) activation, and anti-inflammatory actions as shown by attenuated levels of inflammatory mediators or several angiogenic growth factors including hypoxia-inducible factor 1α, interleukin (IL)-8, and vascular endothelial growth factor. Because we have established an animal model of colitic cancer, which developed dysplasia and cancer similar to human UC, and have found transgelin as a potential biomarker for colitic cancer using this animal model (9), in this study, we hypothesized whether omeprazole, the first PPI targeted for inhibiting gastric acid secretion, could play a chemopreventive role based on its anti-inflammatory or antimutagenic actions beyond acid suppression.

Materials and Methods

Animal model for colitic cancer

Six-week-old female C57BL/6 mice (Charles River Japan) were fed sterilized commercial pellet diets (Biogenomics) and sterile water *ad libitum*, and housed in an air-conditioned biohazard room at a temperature of 24°C. One group of 12 mice, a control group (group 2), was exposed to 15 cycles of dextran sulfate sodium (DSS, molecular weight 40,000; ICN Pharmaceuticals), with each cycle consisting of 7 days of 0.7% DSS w/v in the drinking water, followed by 10 days of sterilized ordinary tap water (Fig. 1A). A second normal control group (group 1) was given ordinary tap water throughout. A third group (group 3) was given daily i.p. injections of omeprazole (AstraZeneca Pharmaceutical) on the same basis as group 2. Animals were handled in an accredited animal facility in accordance with Association for Assessment and Accreditation of Laboratory Animal Care International policies. A dose of 10 mg/kg of omeprazole, i.p. injection, was determined based on our preliminary studies composed of the changes of electron spin resonance measurement, the capability of heme oxidase-1 induction, the degree of apoptosis, and the levels of serum gastrin after omeprazole administration.

Gross and histopathologic evaluations

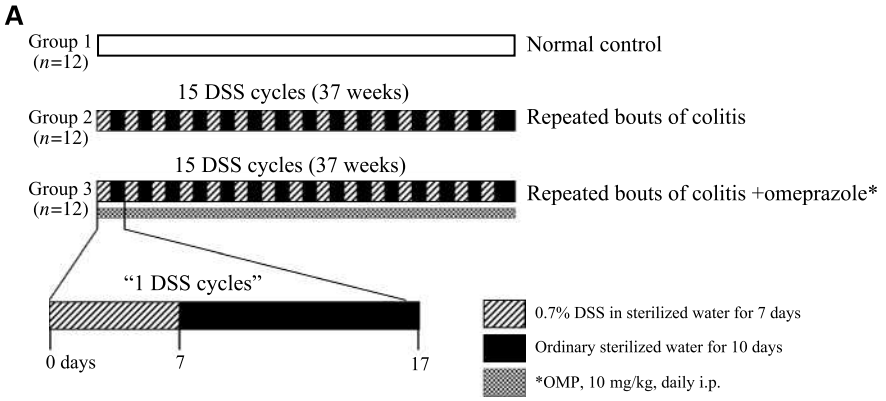
Following sacrifice, the colons were removed. Each dissected colon was spread onto a plastic sheet, fixed in 10% buffered formalin for 4 hours, and prepared for paraffin tissue slides. The paraffin sections were stained with H&E, and the severity of colitis was graded on a scale of 0 to 3, with 0 for absence of inflammation, 1 for focal inflammatory cell infiltration, 2 for gland loss with inflammatory cell infiltrations, and 3 for ulcerations. The tumors were counted upon gross examination (Fig. 1B) and their sizes were measured. The pathology of each tumor was evaluated by two pathologists unknown to the group. Colitis-associated colon neoplasms were analyzed microscopically and diagnosed as low-grade dysplasia, high-grade dysplasia, and adenocarcinoma. Tumor incidence was calculated as the number of tumor-bearing mice divided by the total number of mice, whereas tumor multiplicity was calculated as the number of tumors divided by the number of tumor-bearing mice.

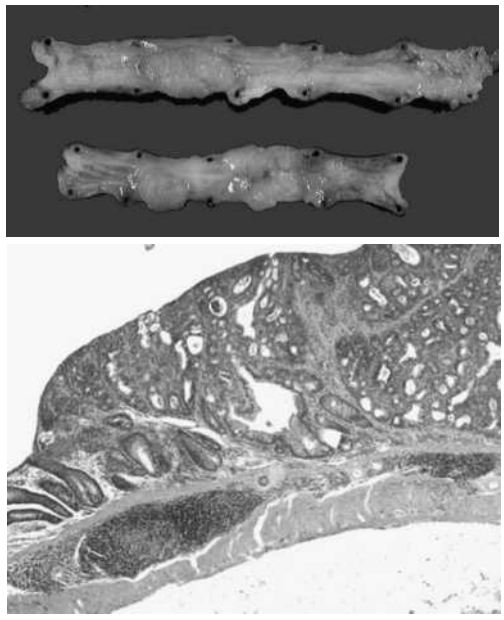
Measurement of tumor necrosis factor-α, IL-6, nitric oxide, gastrin, and thiobarbituric acid–reactive substance levels

To detect colitis activity, the levels of nitric oxide (NO) and tumor necrosis factor-α (TNF-α) in the serum were measured. All samples were measured for TNF levels and each sample was analyzed in triplicate, taking the mean of the three determinations. To measure the level of NO, obtained bloods were centrifuged for 10 minutes before being stored at −70°C. Serum nitrite concentrations were determined by colorimetric assay based on the Griess reaction using a Nitrate/Nitrite Colorimetric Assay Kit (Cayman Chemical, Co.) and ELISA was applied to detect the titer of the gastrin (mouse gastrin ELISA kit; Cusabio Biotech), TNF-α in serum, and IL-6 levels in tissue homogenates (R&D Systems) according to the instructions of the manufacturer. Tissue levels of thiobarbituric acid–reactive substance (TBA-RS) were measured with an assay kit for MDA levels from OXIS (Bioxytech MDA 586; Percipio Biosciences, Inc.).

Reverse transcription-PCR for inflammatory cytokines and matrix metalloproteases

Total RNA were extracted from tissues using an RNeasy mini kit (Qiagen, Inc.). Primers used for inflammatory cytokines were as follows: 5-TCT CTT CAA GGG ACA AGG CTG-3 and 5-ATA GCA AAT CGG CTG ACG GT-3 for *TNF-α*, 5-TTG TTG CCA TCA ATG ACC CC-3 and 5-TGA CAA AGT GGT CGT TGA GG-3 for *GAPDH*. Primers for matrix metalloproteases (MMP), MT-MMPs, and tissue inhibitor of metalloproteinases (TIMP) were as follows: 5′-AGA TCT TCT TCT TCA AGG ACC GGT T-3′ and 5′-GGC TGG TCA GTG GCT TGG GGT A-3′ for *MMP-2*, 5′-GAT TCT TTC ATT TTG GCC ATC TCT TC-3′ and 5′-CTT CCA GTA TTT GTC CTC TAC AAA GAA-3′ for *MMP-3*, 5′-TAC TGG ACT GAT GAT GAG GA-3′ and 5′-AGC ACA AGG AAG AGG GAG AC-3′ for *MMP-7*, 5′-GTT TTT GAT GCT ATT GCT GAG ATC CA-3′ and 5′-CCC ACA TTT GAC GTC CAG AGA AGA A-3′ for *MMP-9*, 5′-ATT TGG TTC TTC CAA GGT GCT CAF T-3′ and 5′-CCT CGG AAG AAG TAG ATC TTG TTC T-3′ for *MMP-11*, 5′-ATG ATC TTT AAA GAC AGA TTC TTC TGG-3′ and 5′-TGG GAT AAC CTT CCA





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Tumor incidence and multiplicity according to group

Groups	Treatments	Tumor Incidence (%)	Tumor multiplicity
Group 1	None	0/10 (0.0)	-
Group 2	Repeated DSS	9/12 (75.0)	1.57±0.5
Group 3	Repeated DSS+Omeprazole	3/12 (25.0)*	1

* $P<0.001$

Fig. 1. Repeated colitis-induced tumorigenesis as an animal model for colitic cancer. A, overview of experimental protocol of DSS-induced colitic cancer model in mice. Control mice were fed a normal diet and given ordinary sterilized tap water (group 1). Colitis was induced by imposing 15 cycles (255 d in total) of DSS in the drinking water, with each cycle consisting of 7 d of 0.7% DSS, followed by 10 d of ordinary sterilized tap water (group 2). In group 3, mice were treated with omeprazole, daily i.p. under the same conditions as group 2. B, gross appearance. Colon tumors, protruding mass scattered along the colon or single mass, were observed in 9 of 12 (75.0%) mice in group 2. Representational gross and pathologic pictures are shown (magnification, ×100). C, incidences of colon tumors in colitic cancer models according to group. Statistically significant decreases in tumor incidence were noted in group 3 compared with group 2 ($P < 0.001$).

GAA TGT CAT AA-3′ for *MMP-13*, 5′-CTG ACG ATC TCG AGT GGA ACT AAA CCC CAG AGT CC-3′ and 5′-CTG AAG CTA AGC TTG GTC CGA GAC CAC CGG GTC AG-3′ for *MT1-MMP*, 5′-CGC CAA GAC GGT CGT TTT GTC TTT T-3′ and 5′-GGG CTC CAG GTT CGG TTC T-3′ for *MT2-MMP*, 5′-GCA GTG AAG AGT TTC TCA TC-3′ and 5′-TCA TCG GGC CCC AAG GGA TCT-3′ for *TIMP-1*, 5′-GCA GGA AAG GCA GAA GGA GAT-3′ and 5′-TTA CGG GTC CTC GAT GTC AAG-3′ and 5′-GCA GAT GAA GAT GTA CCG AGG-3′ for *TIMP-2*. The amplifications were done in 50 μL reaction volumes containing 10× reaction buffer (Promega Korea, Seoul), 1.5 mmol/L of $MgCl_2$, 200 mmol/L of deoxynucleotide triphosphates, 1 mmol/L of each primer, and 2.5 units of Taq DNA polymerase

(Promega) using a Perkin-Elmer GeneAmp PCR System 2400. Each cycle consisted of denaturation at 95°C for 1 minute, annealing at 55°C for 45 seconds, and amplification at 72°C for 45 seconds. The reverse transcription-PCR–derived DNA fragments obtained with 25 PCR cycles were subjected to electrophoresis on a 1.5% agarose gel. The results were also scanned and quantified by densitometry (Appraise; Beckman-Coulter Korea).

Immunohistochemical staining

Cyclooxygenase-2 (COX-2) and inducible NO synthase (iNOS) expression were evaluated in colon tissues. After paraffin blocks were dewaxed and rehydrated with graded alcohol. These tissue sections were then heated in pressure jars filled with 10 mmol/L of citrate buffer using a microwave for 10 minutes. Afterwards, slides were cooled in water for 15 minutes and then washed in phosphate buffer saline. The slides were incubated overnight with the primary antibody. The primary antibodies were specific rabbit polyclonal antibody of COX-2 and iNOS (Santa Cruz Biotechnology). Each antibody was diluted 1:100. After incubation, the subsequent reactions were formed using an Envision kit (DakoCytomation). Finally, the slides were incubated with 3,3′-diaminobenzidine (DakoCytomation) and counterstained with hematoxylin (Sigma). Each stain result was subdivided into three groups according to the percentage of positive stains in the tissue block (0, <5%; 1, 5-50%; and 2, >50%) and analyzed.

Terminal deoxynucleotidyl transferase–mediated nick-end labeling staining

Apoptosis was visualized with terminal deoxynucleotidyl transferase FragEL DNA fragmentation detection kit (Oncogene Research Products). After routine deparaffinization, rehydration, and washing in 1× PBS (pH 7.4), tissues were digested with proteinase K (20 μg/mL in 1× PBS) for 20 minutes at room temperature and washed. Afterwards, tissues were incubated in equilibration buffer for 10 minutes and were treated with terminal deoxynucleotidyl transferase enzyme at 37°C for 1 hour. To determine the apoptotic index in each group, we first scanned terminal deoxynucleotidyl transferase nick-end labeling (TUNEL)–immunostained sections under low-power magnification (×100) to locate the apoptotic hotspots. The apoptotic index at ×400 field was then scored by counting the number of TUNEL-positive cells. At least five hotspots in a section were selected and average count was determined. Data were expressed as a mean percentage of total cell numbers.

Cell cultures and assay for cell viability and growth

The human colon cell line HT-29 was obtained from American Type Culture Collection and cultured in RPMI culture medium (Life Technologies, Inc.) containing 10% fetal bovine serum and supplemented with penicillin and streptomycin (both at 2%). Cells were maintained in a humidified incubator at 37°C in the presence of 5% CO_2. Cell survival was measured using a standard methyl thiazoyl tetrazolium (MTT) assay. Briefly, following treatment with 500 μmol/L of omeprazole or 500 μmol/L of omeprazole plus 1 μmol/L of gastrin, cells were grown overnight, then trypsinized, plated out into 96-well plates as replicates and incubated overnight in growth medium. The following day, the medium was replaced with RPMI 1640 culture medium supplemented with 1% serum, and the cells were grown for an additional 96 hours. Then, the medium was replaced with fresh medium containing MTT at 1 mg/mL and incubated for 4 hours. The medium was removed, the incorporated MTT dissolved in DMSO, and the absorbance was read at 550 nm. To perform cell growth studies, HT-29 cells at 70% confluence were collected by trypsinization, seeded in 96-well plates (5×10^4 cells per well), and allowed to adhere in the presence of standard culture medium. After 24 hours, cells were washed with PBS and serum-free medium was added. The next day, 1 μmol/L of gastrin (Sigma) or 1 μmol/L of gastrin plus 500 mmol/L of omeprazole was added to the culture medium for 48 hours. Western blot analyses for caspase-3 were done on cell lysates. Fractions containing identical levels of proteins were separated by SDS-PAGE and analyzed by Western blotting with the antibodies caspase-3 and β-actin (both from Santa Cruz Biotechnology).

Statistical analysis

Results are expressed as the mean ± SD. The data were analyzed by one-way ANOVA, and the statistical significance between groups was determined by Duncan's multiple range test. Statistical significance was accepted at $P < 0.05$.

Results

Establishment of an animal model for colitic cancer and cancer prevention with omeprazole treatment

Repeated inductions of mild colitis provoked with 0.7% DSS in drinking water (15 cycles in total, each cycle is 17 days composed of 7 days of administration of 0.7% DSS in drinking water followed by 10 days of sterilized tap water) led to the significant development of colorectal tumors. In spite of the absence of carcinogens, colon tumors were generated in 9 of 12 (75.0%) mice only with repeated bouts of mild colitis as shown in the protocol, assuring that a course of chronic colitis with alternating relapse and remission was the link between chronic inflammation and carcinogenesis (Fig. 1A and B). Compared with animal models from other investigators who induced tumors with the administration of azoxymethane [a genotoxic colonic carcinogen, followed by DSS in the drinking water (ref. 14), in which cases the tumor developed mostly on the anal side and spread in the proximity], in our model, tumors developed in the whole colon with mass-forming type features without any prevalent locations (Fig. 1B). Omeprazole treatment induced hypergastrinemia, whereas groups 1 and 2 did not. On pathology, tubular adenoma or moderately differentiated

adenocarcinoma was noted and some cancers invaded deeper than the submucosal layer (Fig. 1B). Group 3 received both repeated inductions of colitis and daily administration of omeprazole (10 mg/kg, i.p.), and developed colon tumor in only 3 of 12 (25.0%) mice, which was statistically significantly decreased compared with group 2 ($P < 0.001$; Fig. 1C), suggesting that omeprazole exerted significant cancer-preventive actions in colitis-associated tumorigenesis.

Footprint of inflammation on colitis-induced tumorigenesis and attenuated inflammatory mediators with omeprazole treatment

To know whether TNF-α, a key cytokine known to be engaged either in the complication of IBD or the development of colitic cancer (6), contributed to our model of colitic tumorigenesis, serum and tissue levels of TNF-α were measured in all groups. As a result, group 2, a control group that underwent repeated inductions of colitis, showed significantly increased levels of serum TNF-α ($P < 0.01$) as well as tissue TNF-α ($P < 0.01$; Fig. 2A). Pooled expression levels of TNF-α mRNA in colon tissues from 10 nontumorigenic mucosa obtained near the location of the tumor were significantly increased in group 2 compared with group 1 (Fig. 2B), signifying that colitic tumorigenesis might be associated with inflammatory flare-ups and oxidative assaults imposed by repeated inductions of colitis. In addition to TNF-α, the serum level of NO, another key biomarker reflecting oxidative stress and degree of colon inflammation, and TBA-RS, reflecting the levels of lipid peroxide formation, were significantly increased in group 2 compared with group 1 (Fig. 2C). However, chronic administration of omeprazole was very effective in either lowering TNF-α or decreasing the oxidative markers of serum NO and colon TBA-RS levels, showing statistically significantly lower levels compared with group 2 ($P < 0.05$). Furthermore, we performed multiplex RNase protection assays for inflammatory cytokines using RNA extracted from pooled samples to document the implication of inflammatory cytokines in colitis-associated carcinogenesis (Fig. 2D). As shown in Fig. 2F, the expressions of TNF-α, IL-6, IFN-γ, and migration-inhibitory factor were apparently increased in group 2 compared with group 1, suggesting that inflammation and oxidative stress might lead to colitis-associated carcinogenesis.

Attenuated transcript levels of cytokines, chemokines, and MMP with omeprazole treatment

For immunohistochemistry, we stained all the animal tissue samples using COX-2 and iNOS antibodies. The immunohistochemical expressions of COX-2 (Fig. 3A) and iNOS (Fig. 3B) were significantly increased in tissue adjacent to tumors, infiltrating inflammatory cells and smooth muscles of the muscularis mucosa and proper muscle layers underneath tumors in cases of COX-2 immunostaining, and epithelial cells and inflammatory cells in cases of iNOS immunostaining. However, the mean scoring for these expressions were significantly attenuated in group 3 compared with group 2, suggesting that the attenuated tumorigenic outcomes of group 3 were based on the anti-inflammatory actions of omeprazole. Because previous RNase protection assays implicated IL-6 in group 2, we measured the levels of IL-6 according to group. As a result, the levels of mucosal IL-6 were significantly increased in group 2, but their levels were significantly decreased in group 3 ($P < 0.01$; Fig. 3C). In addition to the changes in inflammatory cytokines, we checked the expression of MMPs according to group because MMPs are known to be engaged principally in colitic cancer (15). Increased expressions of MMP-3, MMP-9, MMP-11, MMP-13, and TIMP-1 were noted in group 2 compared with group 1 (Fig. 3D), suggesting that increased MMPs contributed to the development of colitis-associated carcinogenesis. Significant anti-protease activities were exerted with omeprazole administration as shown by the statistically significantly attenuated expressions of MMP-9, MMP-11, and MT1-MMP.

Increasing apoptosis in colitic cancer tissues with omeprazole treatment

Because attenuated or abolished apoptotic executions in tumor sections are a generally acknowledged finding in colitic cancer models as well as in sporadic colorectal cancer, showing that deranged apoptotic activities might be fundamentally prerequisite mechanisms for tumorigenesis and providing opportunities for tumor expansion, we evaluated the apoptotic activity of colitic tumor tissues and neighboring nontumorous tissues according to group. Attenuated apoptotic activities were noted in tumor tissues from group 2 as seen in Fig. 4A. However, increased apoptotic activities were observed even in tumor tissues from group 3 (Fig. 4A, bottom), signifying that considerable levels of apoptotic activities even in tumor tissue might be one of the fundamental mechanisms of antitumorigenesis in group 3. Apoptotic index was significantly increased in group 3 compared with groups 1 or 2 ($P < 0.05$; Fig. 4B). If we analyzed apoptotic index only in tumor tissue, the significance might have been very high, but because only three tumors were found in group 3, we presented mean apoptotic indices scored in whole colon tissue comprised of tumors and their neighboring tissue.

Attenuated formation of β-catenin–accumulated crypt in colitic cancer tissue with omeprazole treatment

The expression of β-catenin was evaluated by immunohistochemical staining. As shown in Fig. 5A and B, β-catenin was stained mostly in the membrane of colonocytes in nontransformed mucosa, whereas in transformed glands, the β-catenin was definitely stained in the nucleus in accordance with increased expressions in the cytoplasm and membrane. Different expression patterns of β-catenin were definitely noted in normal mucosa and cancerous mucosa (Fig. 4C). The specificities of β-catenin immunostaining were proven with the omission or addition of β-catenin antibody (Fig. 4C, top). An important finding

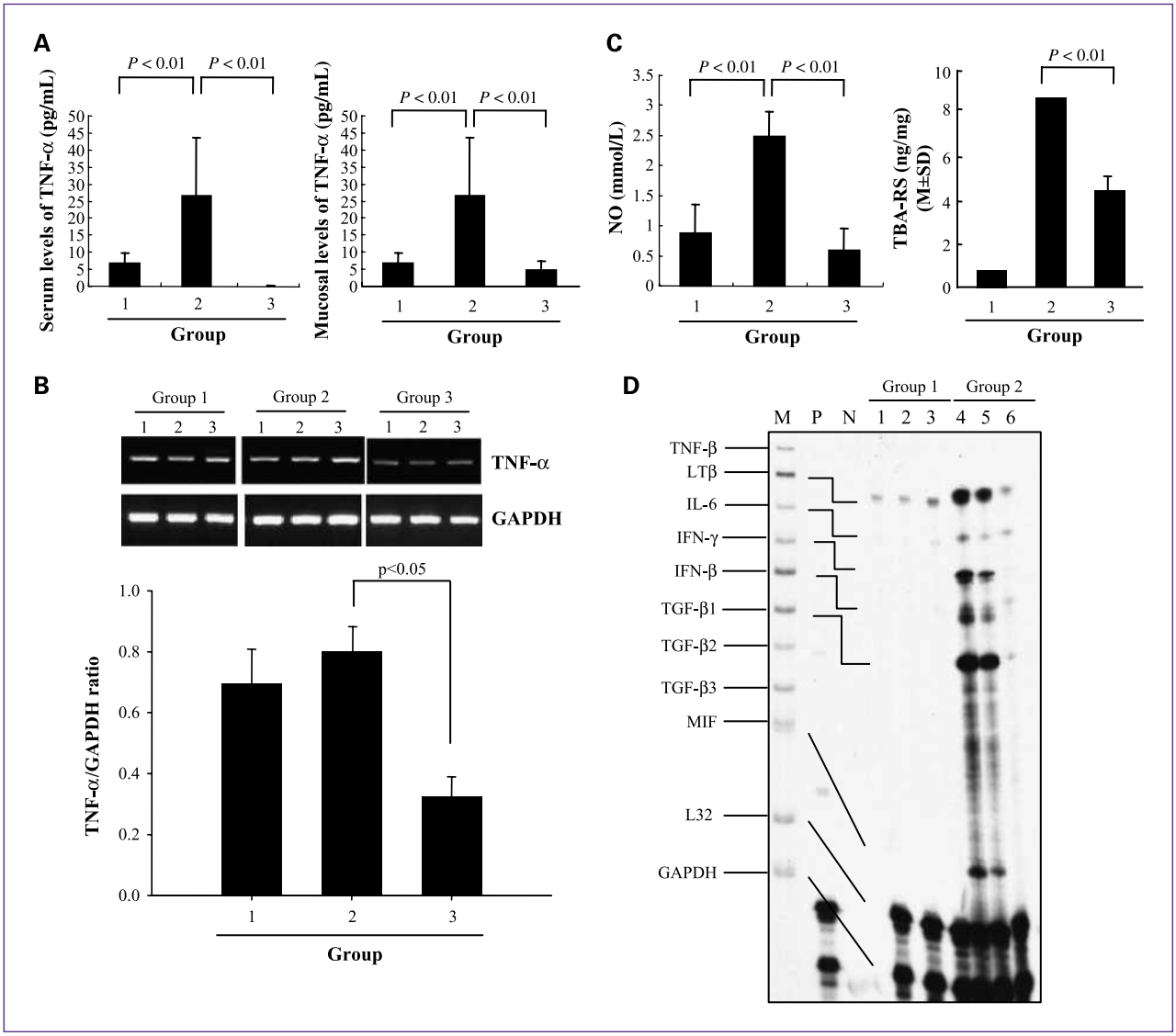

Fig. 2. Changes of TNF-α, NO, and TBA-RS levels according to group. A, serum and tissue TNF-α levels. The serum TNF-α levels were significantly increased in group 2, whereas their levels were significantly decreased in group 3 ($P < 0.01$). Similar to serum levels, the tissue TNF-α levels were significantly increased in group 2, whereas their levels were significantly decreased in group 3 ($P < 0.01$). B, comparison of tissue TNF-α mRNA according to group. The mRNA expression of TNF-α in group 2 was slightly increased, but their levels of expression were significantly decreased in group 3. C, serum levels of NO and tissue levels of TBA-RS. The mean serum NO levels and tissue TBA-RS levels of group 2 were significantly increased compared with group 1, whereas their mean levels were significantly decreased in group 3 compared with group 2 ($P < 0.01$). D, a multi-probe RNase protection assay for mouse cytokine RNase using a mCK-3b (PharMingen) kit was done. M, molecular size marker; P, positive control RNA; N, negative control. L32 and GAPDH served as housekeeping genes.

regarding β-catenin in colitic cancer is shown in the middle panel of Fig. 4C (bottom), the so-called β-catenin–accumulated crypt (BCAC). Usually, the number of aberrant crypt foci (ACF) are counted after staining the colon tissue with a methylene blue solution to reflect the potentiality of premalignant lesions (16), however, BCAC have been acknowledged as a more specific marker for potential cancerous risk compared with ACF (17). Because debates still exist about the real significance of ACF, we measured the number of BCAC in the current study. When we counted the mean numbers of BCAC in ×100 magnified field of microscopy according to group, statistically significant increases in mean BCAC numbers were noted in group 2 ($P < 0.001$; Fig. 4D). On the other hand, the mean numbers of BCAC were significantly decreased in group 3 compared with group 2 ($P < 0.01$), showing that omeprazole afforded significant antimutagenic actions.

Blocking the trophic effect of gastrin on colon cancer with omeprazole treatment

Gastric acid inhibition with longstanding administration of PPI is associated with hypergastrinemia. Therefore,

hypergastrinemia which developed with long-term PPI use increased the risk of colorectal cancer because gastrin imposes trophic effects on colon epithelium, rendering hyperproliferating chances on colon mucosa (18). As anticipated, daily administration of omeprazole for longer time periods (group 3) provoked significantly increased levels of serum gastrin compared with group 2 ($P < 0.01$; Fig. 5A). Therefore, we hypothesized that PPI should abolish these trophic privileges of gastrin on colon epithelial cells to achieve antitumorigenic outcome. It has been reported that PPI could induce selective apoptosis in cancer cells (13, 19), whereas omeprazole (500 μmol/L) induced significant levels of cytotoxicity in cancer cells as shown in Fig. 5B. However, 1 μmol/L of gastrin pretreatment significantly decreased the cytotoxicity of omeprazole. Because the cytotoxicity of omeprazole against cancer cells was

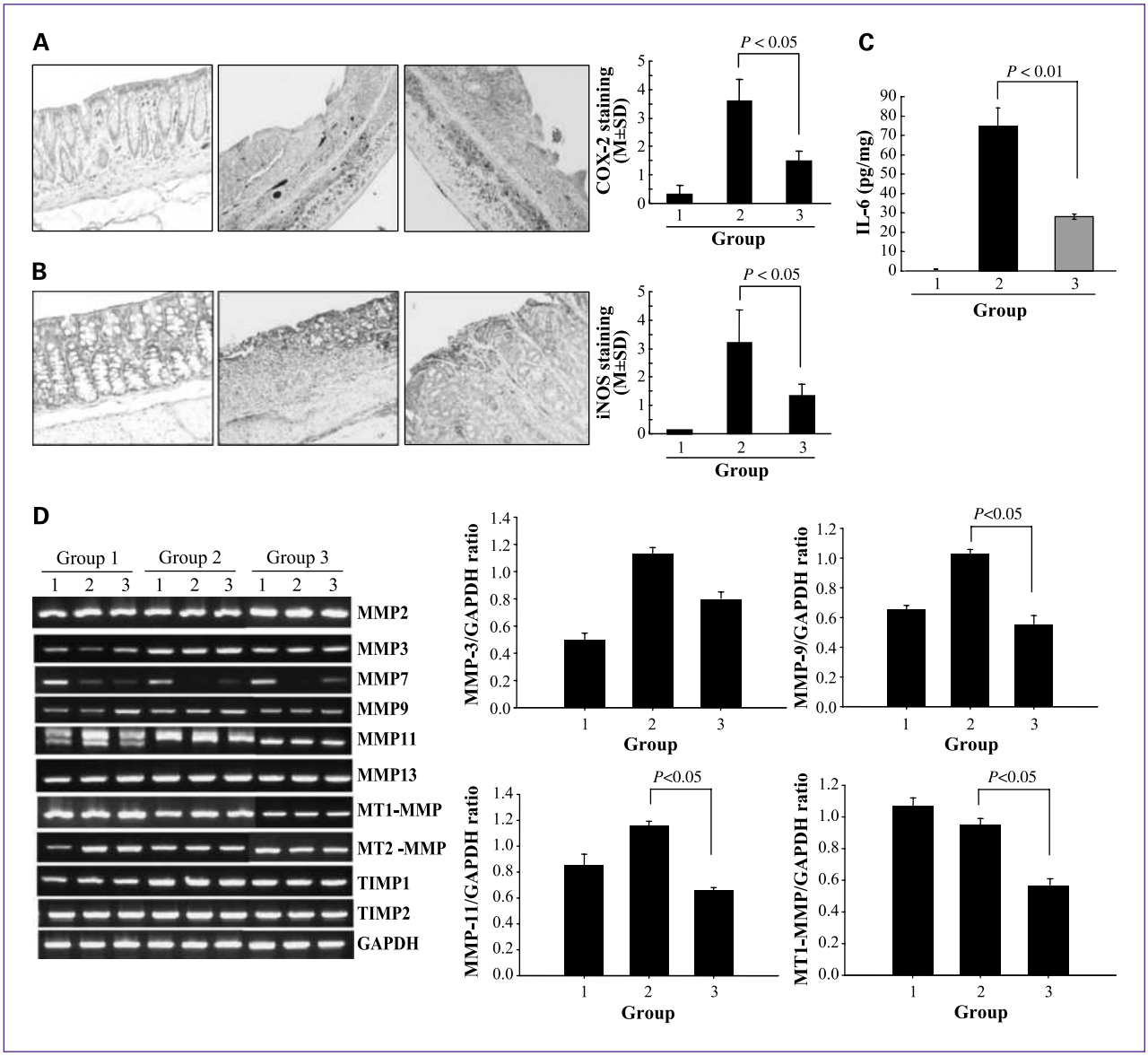

Fig. 3. Changes of inflammatory cytokines and MMPs according to group. A, COX-2 expression. COX-2 was scantly observed in group 1, whereas increased expression was noted in inflamed colon mucosa, submucosa, and muscle layers of group 2. COX-2 expression was more prominently increased in muscularis mucosa and proper muscle layer underneath tumor-bearing mucosa. The mean scoring of COX-2 expression according to group showed that statistically decreased levels of COX-2 were noted in group 3 compared with group 2 ($P < 0.05$). B, iNOS expression. iNOS was not detected in group 1, but the expression of iNOS was apparently increased in group 2. In group 3, iNOS expression was increased in apical sites of tumorigenic mucosa. The mean scoring of iNOS expression according to group showed that statistically significantly decreased levels of iNOS were noted in group 3 compared with group 2. C, colonic levels of IL-6. IL-6 were barely detected in colon mucosa of group 1, but the levels were considerably increased in group 2. However, the levels of IL-6 in group 3 were significantly attenuated compared with group 2 ($P < 0.05$). D, MMP expression: reverse transcription-PCR and their mean densitometric analyses. MMP-3, MMP-9, and MMP-13 were increased in group 2 compared with group 1, but the expressions of MMP-9, MMP-11, and MT1-MMP in group 3 were significantly decreased compared with group 2.

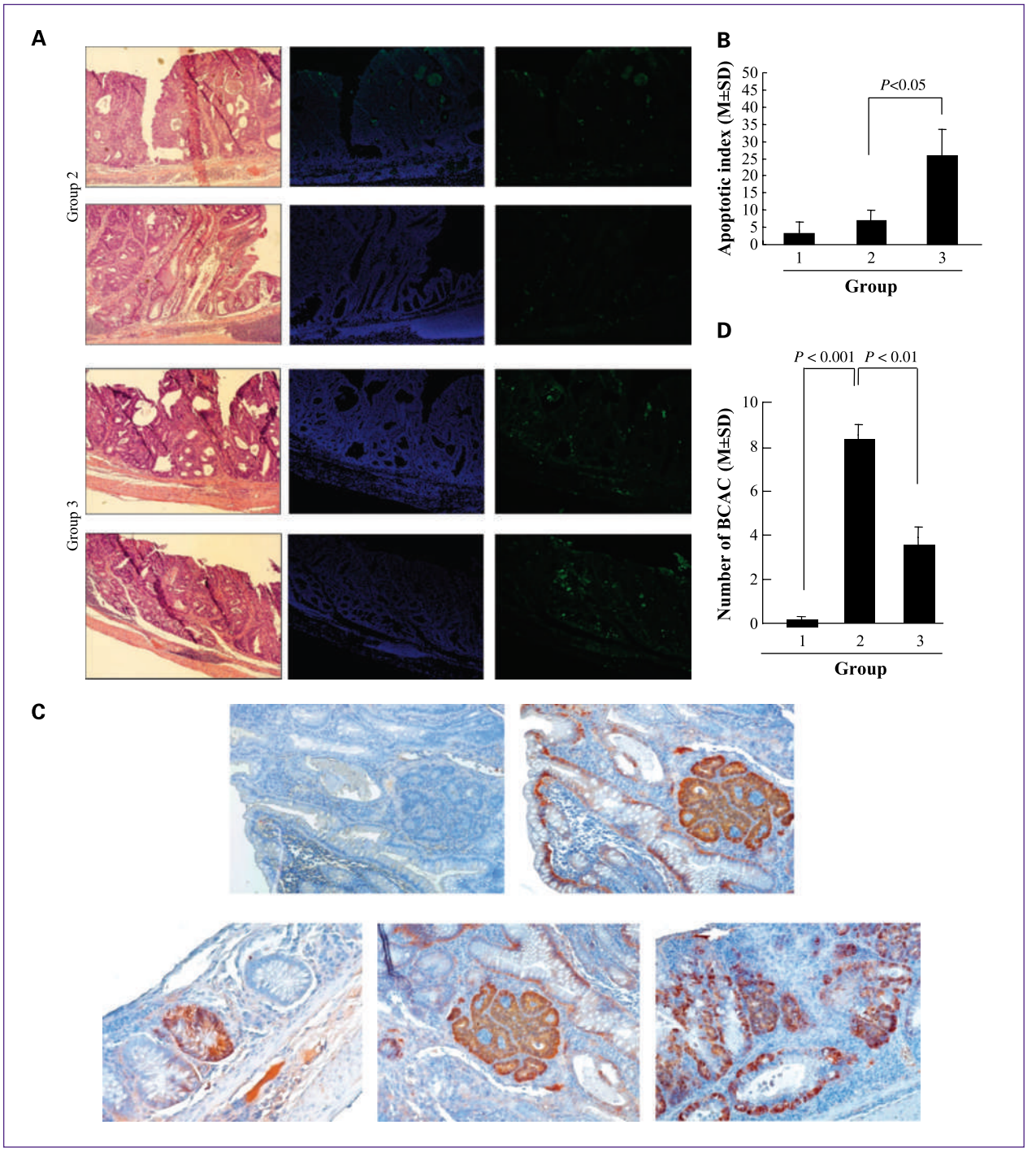

Fig. 4. Apoptosis according to group. A, TUNEL findings in tumors of group 2 and group 3. The TUNEL-positive cells, apoptotic cells, were scantly detected in tumor tissues of group 2, whereas TUNEL-positive cells were clearly observed even in tumor tissues in addition to nontumor tissues of group 3. B, apoptotic index (AI) according to group. Apoptotic index was calculated as the mean number of positive cells under ×100 magnified field of microscopic examination. Apoptotic index was significantly increased in group 3 compared with group 2 ($P < 0.05$). C and D, BCAC and the influence of gastrin. C, immunostaining of β-catenin and BCAC. Top, β-catenin staining was prominently noted in the membrane and cytoplasm of colonocytes in normal colon mucosa, whereas aberrantly increased expressions in either cytoplasm or nucleus were noted in BCAC, which is a kind of biomarker for transformation. Left, to prove the specificity of β-catenin staining, negative controls showed no addition of β-catenin antibody during immunostaining procedures. Bottom, as tumorigenesis progressed, more prominent findings of nuclear translocation of β-catenin and increased expressions were observed. D, mean numbers of BCAC according to group. Because BCAC could predict the risk of malignant transformation of aberrant crypt better than ACF (22), we counted the whole numbers of BCAC according to group. BCAC was statistically increased in group 2, whereas the mean numbers were significantly decreased in group 3, suggesting that omeprazole hindered the development of BCAC.

induced through apoptosis, we checked the executor of apoptosis, caspase-3 (Fig. 5C). Significant cleavage of capsase-3 was noted in the presence of 500 μmol/L of omeprazole, but its cleavage was somewhat attenuated during gastrin pretreatment, signifying that gastrin could attenuate the cytotoxicity of PPI by decreasing apoptosis. These results suggested that omeprazole could block the trophic effects of gastrin; therefore, to document this, we checked cell proliferation after 48 hours of cell cultures. Compared with the gastrin-treated group, cell proliferation was significantly attenuated in the presence of omeprazole ($P < 0.05$), suggesting that PPI could offset the trophic action of gastrin on colon cells (Fig. 5D). Taken together, omeprazole could exert significant levels of anti-inflammatory actions in addition to the appropriate execution of apoptosis and hindrance of β-catenin accumulation, based on which significant prevention in colitis-induced tumorigenesis could be achieved (Fig. 6).

Discussion

Because several studies have suggested that chronic inflammation leads to carcinogenesis, the efficient suppression of chronic inflammation could prevent inflammation-associated cancer (20). For instance, anti-inflammatory drugs or agents such as histone deacetylator inhibitor, naturally occurring phytoceuticals, and other synthetic chemicals could provide chemopreventive effects in models of inflammation-related tumorigenesis (21). In the current study, we added novel evidence that omeprazole, administered at longer time periods, could reverse the progression of inflammation-initiated or promoted carcinogenic step. In addition to our article and other previous publications showing that PPIs could be applied for imposing selective apoptosis of cancer cells (13, 19), anti-angiogenic actions related to *Helicobacter pylori* infection (12), and anti-inflammatory actions with significant induction of heme oxidase-1 (10, 11), we have *in vivo*

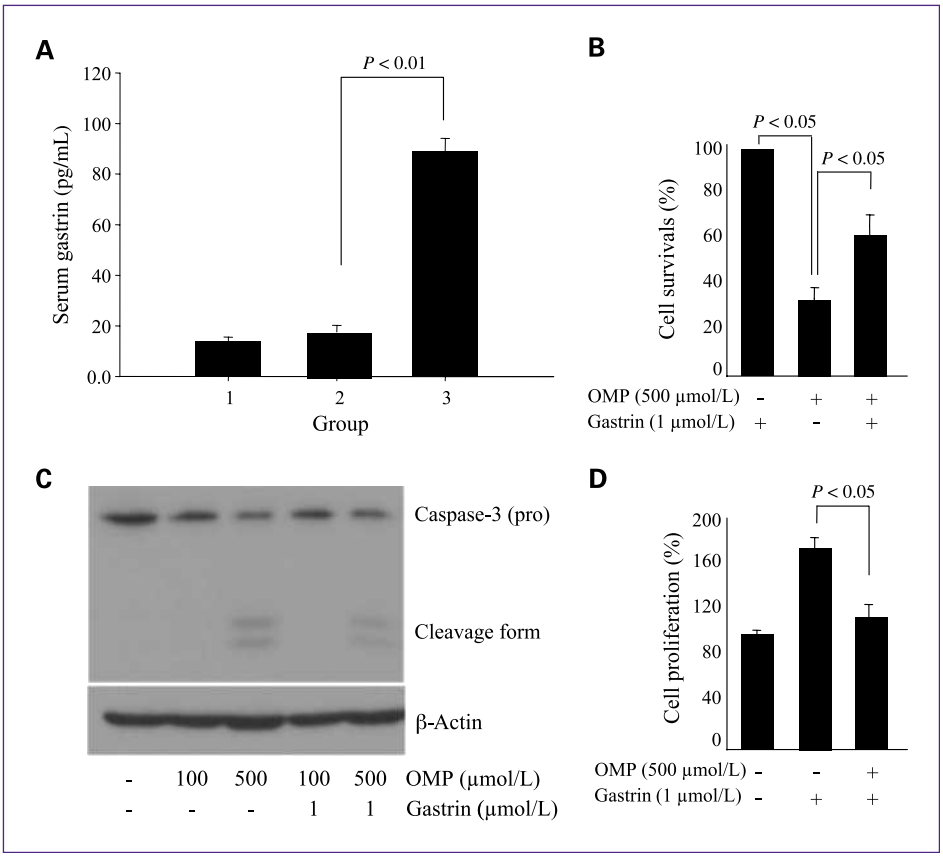

Fig. 5. A, serum gastrin according to group. The mean levels of serum gastrin were significantly increased in group 3 compared with either group 1 or group 2, suggesting that chronic administration of omeprazole was associated with hypergastrinemia. B, cell survival after either omeprazole alone or cotreatment of omeprazole and gastrin MTT assays were done to count the viable cells (HT-29 colon epithelial cells). Treatment with 500 μmol/L of omeprazole caused a significant decrease in cell viability. However, pretreatment with 1 μmol/L of gastrin before omeprazole treatment significantly improved cell survival, suggesting that gastrin rescued the cytotoxicity of omeprazole. C, Western blot for caspase-3. Active cleavage of caspase-3 was noted in the presence of 500 μmol/L of omeprazole, whereas gastrin pretreatment attenuated the active cleavage of caspase-3 imposed by omeprazole. D, cell proliferation (48 h) compared with the gastrin alone group, cotreatment of omeprazole and gastrin significantly decreased cell growth of HT-29, suggesting that omeprazole abolished the benefits of gastrin's proliferative action.

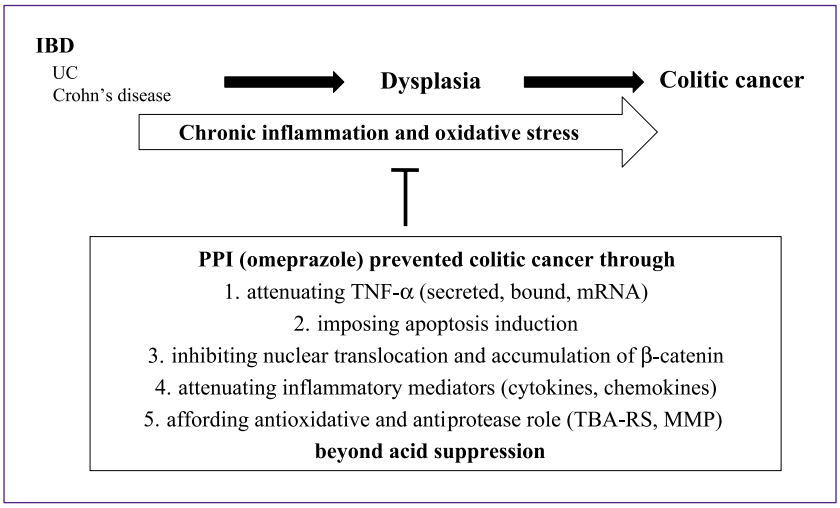

Fig. 6. Connection between chronic inflammation and carcinogenesis and their blocks with omeprazole treatment. The fact that repeated bouts of inflammation resulted in tumorigenesis without any intervention of carcinogen signifies that the early and appropriate intervention of anti-inflammatory and antimutagenic strategy could lead to efficient ways of chemoprevention in longstanding IBD. Omeprazole, even though its primary indication is the suppression of gastric acid secretion, bestowed significant levels of anti-inflammatory and antioxidative actions in addition to apoptosis and the hindrance of β-catenin accumulation in colitic cancer model, through which significant cancer-preventive results were achieved.

evidence to show, for the first time, that PPI could exert cancer-preventive actions based on anti-inflammatory, antimutagenic, and antioxidative actions. There have been several reports in the literature eliciting the anti-inflammatory actions of PPI beyond its fundamental action of acid suppression—lansoprazole for ischemic-reperfusion intestinal injuries, indomethacin-induced rat enteritis (22) and for a rhinoviral infection (23), heme oxidase-1 induction by omeprazole (11), IL-1β inhibition by *v* type-ATPase inhibitors, bafilomycin A, or FR 177995 (24), and revaprazan for indomethacin-induced gastropathy (25).

The next question to be raised will be the possible proliferative fears of colon cancer risk with hypergastrinemia caused by PPI. However, the use of PPIs in clinical practice does not measurably increase the risk of colorectal cancer (26, 27), supporting our results that the trophic actions of increased gastrin also could be blocked with PPI. In addition, it has been reported that endogenous elevation of the serum gastrin hormone to five times the normal levels did not show trophic effects on murine colon tumor cells. Because the expression of iNOS, TNF-α, and COX-2 is usually increased in the colonic tissues of patients with IBD and human colon adenomas (28), in contrast, the inhibition of these molecules could warrant the suppression of colitis-related colon carcinogenesis (29). We found that repeated colitis increased the expression of NO and TNF-α, which ultimately led to tumorigenesis in the affected colon, but omeprazole treatment attenuated the expressions of NO and TNF-α efficiently.

In contrast to sporadic colorectal cancer, in which cancer develops from polyps with a so-called "adenoma-carcinoma" sequence, the premalignant histologic change in IBD is usually referred to broadly as dysplasia rather than adenoma, therefore, the "dysplasia-carcinoma" sequence. The risk of developing colorectal cancer in patients with UC is higher in patients with extensively involved entire colon lesions (30) and very often in nonpolypoid lesions with dysplasia (31). In the current study, our animal model could represent these pathologic features as seen in human colitic cancer. Because colitis-associated cancer arose in the setting of chronic inflammation, factors associated with inflammation such as oxidative stress, cytokine or chemokine increase, and MMP activation could be identified, which are somewhat different molecular alterations and histologic features compared with that seen in sporadic colorectal cancer (32). That is, a high frequency of p53 mutations in inflamed tissues than in uninflamed tissues of UC patients, increased expressions of COX-2, iNOS, IFN-inducible gene 1-8H in mucosa from patients with active UC, and increased reactive oxygen species in UC mucosa (33, 34). However, no mutation in β-catenin was assessed with direct sequencing, no mutation in either p53 or APC assessed with single-strand conformational polymorphisms were noted in our colitis-associated cancer model (9), all genetic changes which were reported to have occurred at very late stages of colitic cancer compared with sporadic colorectal cancer.

Several animal models for colitic cancer have been reported, among which the most widely used are either a rather longer period or repeated administration of DSS (9, 35) or azoxymethane-initiated and DSS-induced colitis promoting two-stage carcinogenesis (14). Relevant to the clinical course of colitic cancer development in IBD patients, the former model of repeated bouts of colitis is quite similar to human disease compared with the latter model. Even though the times for tumorigenesis were reported to be shorter and more convenient in $APC^{min/+}$ of p53-deficient mice than in wild-type animals (36), in the current study, we used C57BL/6 wild-type mice with rather longer periods of repeated colitis using lower concentrations of DSS administration. In a study by Okayasu et al., repeated colitis was induced by administering 3% DSS in mice (35), however, we administered 0.7% DSS in drinking water, necessitating longer time periods of 15 cycles for tumor development. We adopted this latter strategy as a colitic cancer model because the usual life span of a mouse is

around 80 weeks and colitic cancer usually develops after more than 10 years of illness, we administered low concentrations of DSS for longer time periods simulating the clinical course of relapse and remission.

MMPs are a family of Zn^{2+}-containing endopeptidases capable of degrading most components of the extracellular matrix, based on which MMPs are generally implicated in the propagation of inflammation, carcinogenesis, and metastasis (37). Upregulation of MMP-2, MMP-3, MMP-7, MMP-9, and MMP-13 was observed in mice with DSS- or trinitrobenzene sulfuric acid–induced colitis (38, 39). Because MMP-2, MMP-3, and MMP-9 are known to be engaged in colitic cancer (32, 40), the treatment of mice with an inhibitor of MMPs attenuated the severity of experimental colitis (41). Similarly, we observed that omeprazole inhibited the expression of MMPs in DSS-induced colitis and colitic cancer.

The histopathology of dysplasia and cancer in this model is very similar to that seen in the human. In our model, animals developed dysplasia in both flat mucosa and displasia-associated lesion or mass identical to that seen in humans (5, 42). In the human, hyperactivation of β-catenin signaling by mutations in either APC or β-catenin is considered one of the earliest events in the sequence of genetic changes that lead to colon cancer development (43), and sporadic colorectal adenomas and carcinomas showed translocation of β-catenin from the cell membrane to the cytoplasm/nucleus (44, 45). Several agents, such as folic acid, short chain fatty acid (butyrate), ursodeoxycholic acid, and 5-aminosalicylic acid, have been suggested to be useful for the prevention of colorectal cancer in UC (46). In addition, nonsteroidal anti-inflammatory drugs, including COX-2 inhibitors and peroxisome proliferator-activated receptor ligands such as troglitazone and bezafibrate suppressed the development of chemically induced colon carcinoma in rats (47). We could add the novel finding that omeprazole's inactivating action of β-catenin afforded significant levels of cancer prevention compared with these agents. ACF identified in whole-mount preparations of colonic mucosa in rodents, and also recognized in human colon, are now frequently used as effective surrogate biomarkers for the experimental detection of chemopreventive agents against colorectal cancers, but the preneoplastic or precancerous nature of ACF in rodents and humans still remains inconclusive (48). Instead of obscuring the significance of ACF, early appearing BCAC have been described in enface preparations of colonic mucosa in rodents which differ from ACF on many features. Histologic observation showed that BCAC exhibit cellular dysplasia, have higher cellular proliferation, and are more likely to progress to malignant transformation as compared with ACF, leading to the conclusion that BCAC are useful as intermediate biomarkers for colon carcinogenesis in mice.

Taken together, we identified that chronic relapsing inflammation provides enough basis for colitic cancer whereas omeprazole bestowed significant anti-inflammatory, antioxidative, and antimutagenic actions beyond acid suppression (Fig. 6), a much better outcome than previous publications (49) showing that the inhibition of azoxymethane induced colorectal cancer with omeprazole. Therefore, a prospective controlled clinical trial will be required to draw a definite conclusion about the real applicability of omeprazole for cancer-preventive purposes. Even though there is still future work to be done, including the elucidation of the more exact molecular mechanisms of the anti-inflammatory actions of PPI and a proper administration strategy, our study opened the possibility that modification of PPI targeted for cancer prevention would increase the hope of preventing disease in this era of vague biomarkers of colitic cancer and steeply increased incidence of IBD.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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