

Helicobacter pylori-Induced Invasion and Angiogenesis of Gastric Cells Is Mediated by Cyclooxygenase-2 Induction through TLR2/TLR9 and Promoter Regulation¹

Ya-Jen Chang,^{2*} Ming-Shiang Wu,^{2†} Jaw-Town Lin,[†] and Ching-Chow Chen^{3*}

Cyclooxygenase-2 (COX-2) plays a crucial role in *Helicobacter pylori*-associated gastric cancer. In this study, we report that *H. pylori*-induced COX-2 expression enhances the cancer cell invasion and angiogenesis via TLR2 and TLR9, which can be attenuated by the specific COX-2 inhibitor NS398 or celecoxib. The cAMP response element (CRE) and AP1 sites, but not κ B on the COX-2 promoter, are involved in MAPKs-regulated COX-2 expression. Differential bindings of the CREB-1, ATF-2, *c-jun* to the CRE site, and the *c-fos*, *c-jun*, ATF-2 to the AP1 site are demonstrated by DNA affinity protein-binding, supershift, and chromatin immunoprecipitation assays. Activations of these transcription factors were attenuated by different MAPKs inhibitors. The mutants of TLR2, TLR9, or MAPKs inhibited *H. pylori*-induced COX-2 promoter, CRE, and AP-1 activities. MAPKs inhibitors attenuated the *H. pylori*-induced COX-2 mRNA and protein expressions. These results indicate that *H. pylori* acts through TLR2 and TLR9 to activate MAPKs, especially p38, and their downstream transcription factors (CREB-1, ATF-2, *c-jun*, and *c-fos*), resulting in the activations of CRE and AP-1 on the COX-2 promoter. These intracellular networks drive the COX-2-dependent PGE₂ release and contribute to cell invasion and angiogenesis. *The Journal of Immunology*, 2005, 175: 8242–8252.

H*elicobacter pylori* is a Gram-negative bacterium that colonizes the human stomach (1). It has been identified as a major pathogen leading to the development of a wide range of gastroduodenal diseases, including gastritis, duodenal ulcer, gastric ulcer, gastric adenocarcinoma, and gastric mucosa-associated lymphoid tissue lymphoma (2). Eradication of *H. pylori* prevents the development of new cancers or continued growth of occult cancers in patients with early carcinoma (3), suggesting that *H. pylori* infection may play a role not only in the initiation, but also in the promotion of gastric carcinoma. Cyclooxygenase-2 (COX-2)⁴ is the rate-limiting enzyme for the production of prostanoids (prostaglandins and thromboxanes) from arachidonic acid. Up-regulation of COX-2 plays a central role in the inflammatory changes and tissue damages associated with the chronic *H. pylori* infection, and is also involved in the gastric tumorigenesis (4, 5). The expression of COX-2 protein is significantly higher in patients with gastric cancer than those with nonulcer dyspepsia (6). Therefore, COX-2 expression is assumed to play a key role in *H. pylori*-associated gastric cancer in addition to the propagation of gastric inflammation (7). Recent studies have demonstrated that

COX-2-mediated PG biosynthesis affects carcinogenesis via several different mechanisms. Angiogenesis, the formation of new blood vessels from an existing vascular bed, is a key step in tumor growth and metastasis (8). Up-regulations of IL-8, VEGF, angiogenin, urokinase-type plasminogen activator, and matrix metalloproteinase 9 genes by *H. pylori* have been reported to regulate the angiogenesis and invasion of human gastric carcinoma (9, 10). The implication of COX-2 in tumor angiogenesis has also been documented in the colon cancer cells (11). However, the role of *H. pylori*-induced COX-2 in the cell invasion and angiogenesis of gastric cancer cells remains elusive.

Epithelial cells, like macrophages, recognize microbial infections through TLRs, which recognize conserved motifs on pathogens that are not found in higher eukaryotes (12). All TLRs activate a common signaling pathway that culminates into the activation of NF- κ B and MAPKs (13). The involvement of TLR2 and TLR9 in *H. pylori*-induced COX-2 expression via the PKC/c-Src/NF- κ B activation pathway has been reported from this lab (14). Activations of MAPKs by *H. pylori* have been reported and found to regulate the expressions of many genes such as matrix metalloproteinase 7 and IL-8 (15, 16). Therefore, the role of TLR2 and TLR9 in MAPKs activation induced by *H. pylori* leading to COX-2 expression is further investigated in this study.

Our recent study has demonstrated that *H. pylori* strains isolated from gastric cancer patient (HC) induced a higher COX-2 expression than those from gastritis and peptic ulcer. However, the virulence genes of *iceA*, *vacA*, *babA2*, *caga* 3' repeat region and *hrgA* among various *H. pylori* strains failed to show any association with COX-2 expression despite a higher COX-2 induction capability of HC (14). Involvement of cAMP response element (CRE) site in the *H. pylori*-induced COX-2 expression was demonstrated (14). The importance of CRE and/or AP-1 sites in mediating COX-2 transcription, particularly on the murine and human promoter has been reported (17–20). The AP-1 site (–67/–61) on the human COX-2 promoter adjacent to the downstream CRE site (–59/–53) and to the upstream pNFAT site (–76/–68) was first mentioned by

*Department of Pharmacology and †Division of Gastroenterology, Department of Internal Medicine and Primary Care Medicine, College of Medicine, National Taiwan University and National Taiwan University Hospital, Taipei, Taiwan

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² Y.-J.C. and M.-S.W. contributed equally to this work.

³ Address correspondence and reprint requests to Dr. Ching-Chow Chen, Department of Pharmacology, College of Medicine, National Taiwan University, No. 1, Jen-Ai Road, 1st Section, Taipei 10018, Taiwan. E-mail address: ccchen@ha.mc.ntu.edu.tw

⁴ Abbreviations used in this paper: COX, cyclooxygenase; CRE, cAMP response element; HC, *H. pylori* isolate from gastric cancer; HD, *H. pylori* isolate from duodenal ulcer; HS, *H. pylori* isolate from gastritis; MOI, multiplicity of infection; DAPA, DNA affinity protein-binding assay; ChIP, chromatin immunoprecipitation; wt, wild type.

Fresno and colleagues (17) and Flamand and colleagues (18). By probing consensus sequences containing both CRE and AP-1 sites, Dannenberg and colleagues (21, 22) demonstrated that AP-1 components bind to the CRE site. However, the individual role of CRE and AP-1 could not be clearly differentiated because AP-1 components probably bind to the AP-1 and/or CRE sites. In the present study, DNA affinity protein-binding and gel shift assays were performed using probes containing CRE and AP-1 site consensus sequences, respectively. Thus, the components binding to the CRE or AP-1 site could be clearly distinguished.

The investigation of the relationship between *H. pylori* infection, COX-2 overexpression, and gastric cancer development is still lacking. In this study, we explored a novel intracellular network involving TLRs, MAPKs, and transcription factors binding to the CRE and AP1 sites on the human COX-2 promoter, leading to COX-2 expression and PGE₂ release, which in turn promote gastric cancer cell invasion and angiogenesis.

Materials and Methods

Bacterial strains and growth conditions

H. pylori strains were isolated from patients undergoing gastroscopy for the evaluation of upper gastrointestinal symptoms. At the time of endoscopy, two biopsy specimens were taken from the antrum for bacterial culture. Different strains of clinical *H. pylori* isolates from patients with nonulcer dyspepsia with gastritis, duodenal ulcer, and gastric cancer were termed as HS, HD, and HC, respectively. Columbia agar with 5% sheep blood was used for *H. pylori* culture. The bacterial cells were cultured at 37°C in a microaerophilic chamber (Don Whitley) containing 10% CO₂, 5% O₂, and 85% N₂. Bacterial cells were grown for 48 h on Columbia agar plates, which were collected and washed with PBS buffer (pH 7.4). Finally, the bacterial cells were pelleted. Cell pellets were then resuspended in PBS buffer (pH 7.4) and used for the infection experiment (14).

Cell culture and *H. pylori* infection experiments

The human gastric cancer epithelial cell lines AGS (American Type Culture Collection (ATCC) no. CRL-1739) and MKN45 (Japanese Cancer Research Bank no. 0254) were obtained from the ATCC and RIKEN, respectively. Both of them were cultured in RPMI 1640 supplemented with 10% FCS, 100 U/ml penicillin, and 100 µg/ml streptomycin. AGS and MKN45 cells were cocultured with *H. pylori* in the antibiotics-free RPMI 1640 supplemented with 10% FCS. Bacterial cells were resuspended in PBS (pH 7.4) and diluted to the multiplicity of infection (MOI) of 150:1. Cells were cocultured with bacteria for 16 h. Media were collected from the cells cocultured with bacteria from different time points.

AGS cells were transfected with the COX-2-expressing pcDNA3 vector containing a 1.9-kbp cDNA fragment of human COX-2 or pcDNA3 control vector only using liposomal transfection reagent TransFast (Promega) according to the manufacturer's instructions. After 24 h of transfection, cells were replaced in the complete medium with 600 µg/ml G418. Stable G418-resistant transfectants (the COX-2-expressing clone AGS/COX-2 and the control clone AGS/pcDNA3) were selected and expanded.

Preparation of cell extracts and Western blot analysis

After incubation with *H. pylori*, cells were rapidly washed with PBS to remove bacteria and lysed with the ice-cold lysis buffer (50 mM Tris-HCl (pH 7.4), 1 mM EGTA, 1 mM NaF, 150 mM NaCl, 1 mM PMSF, 5 µg/ml leupeptin, 20 µg/ml aprotinin, 1 mM Na₂VO₄, 10 mM β-glycerophosphate, 5 mM sodium-pyrophosphate, 1% Triton X-100). The cell lysate was subjected to SDS-PAGE using 10% for the running gel. The proteins were transferred to the nitrocellulose paper, and Western blot was performed as described previously (23).

PGE₂ release ELISA

PGE₂ was quantified from the collected media using ELISA by a commercial kit from Amersham Biosciences according to the manufacturer's instructions.

In vitro invasion assay

The invasion assay was conducted using the Transwell cell culture chambers (Corning Costar; No. 3422) according to the manufacturer's recommendation with some modifications. Briefly, polyvinylpyrrolidone-free polycarbonate filters (8.0 µm pore size; Nuclepore) were pre-

coated with 5 µg of Matrigel (BD Biosciences) on the upper surface. AGS or MKN45 cells were harvested with 1 mM EDTA and resuspended in the antibiotics-free RPMI 1640 supplemented with 0.1% FBS. The cell suspension (2.5 × 10⁴ cells) was added to the upper compartment of the chamber. Six hours later, cells were cocultured with *H. pylori* after the pretreatment with either NS-398, celecoxib, or PGE₂ for 30 min. After 48 h incubation, the top side of the insert membrane was scrubbed free of cells with a cotton swab. The bottom side was fixed with 3.7% paraformaldehyde and stained with 0.5% crystal violet in 20% methanol. The crystal violet dye retained on the filters was extracted with DMSO and colorimetrically assessed by measuring its absorbance at 590 nm on an ELISA reader (Bio-Tek).

In vitro tube formation assay

HUVECs, between passages 2 and 4, were plated on a 10-cm dish and grown to confluence. The cells were synchronized in 2% serum containing M199 for 24 h before they were plated in 24-well plates (5 × 10⁴/well) coated with 240 µl of Matrigel (diluted 1/1 in serum-free RPMI 1640). The condition media, which were serum-free supernatants collected from either AGS or MKN45 cells infected with *H. pylori* for 16 h, were added to the HUVECs and incubated for 5 h. Subsequently, the cells were fixed by 3.7% paraformaldehyde and visualized with the microscope using a ×10 objective. Endothelial cells form the connective and complete tube-like structures with branches when cultured in the appropriate media. The number of tubes was quantified in six different fields for each culture (24). Quantification of tube formation was shown as relative to the control.

DNA affinity protein-binding assay (DAPA)

Binding of transcriptional factors to the COX-2 promoter DNA sequences was assayed. After coculture with *H. pylori* for the indicated time, nuclear extracts were prepared. The biotin-labeled double-stranded oligonucleotides were synthesized based on the human COX-2 promoter sequence (25). "CRE + AP1 wt" (sense, 5'-biotin-AAGAAACAGTCATTTTCGTCA CATGG-3'; antisense, 5'-CCATGTGACGAAATGACTGTTTCTT-3') contains the CRE and AP1 binding sites; "CRE mut" (sense, 5'-biotin-AAGAAACAGTCATTTGAGCTCATGG-3'; antisense, 5'-CCATGAGCT CAAATGACTGTTTCTT-3') contains wild-type (wt) AP1 and mut CRE; "AP1 mut" (sense, 5'-biotin-AAGAAACAGCTGTTTCGTCCATGG-3'; antisense, 5'-CCATGTGACGAAACAGCTGTTTCTT-3') contains mut AP1 and wt CRE. The sequence underlined means the "core region" for the transcription factor binding sites, and the sequence with italic type means the "mutated base pair." The binding assay was performed by mixing 200 µg of nuclear extract proteins, 2 µg of biotin-labeled DNA oligonucleotides, and 20 µl of streptavidin agarose beads (4%) with 70% slurry. The mixture was incubated at room temperature for 1 h with shaking. Beads were pelleted and washed with cold PBS three times. The bound proteins were separated by SDS-PAGE, followed by Western blot analysis, and finally probed with specific Abs.

Preparation of nuclear extracts and the EMSA

AGS cells cocultured with *H. pylori* for the indicated time were washed with PBS several times, and nuclear extracts were prepared as described previously (14). Oligonucleotides corresponding to the consensus sequences on the human COX-2 promoter, including "AP1 + CRE wt" (5'-AAGAAACAGTCATTTTCGTCCATGG-3'), which contains the AP1 and CRE binding site; "AP1" (5'-AGAAACAGTCATTTTC-3'), which contains the AP1 binding site; "CRE" (5'-CATTTCGTCA CATGG-3'), which contains the CRE binding site, were synthesized, annealed, and end-labeled with [³²P]ATP using T4 polynucleotide kinase. EMSA was performed as described previously (23). When supershift assays were performed, polyclonal Abs specific to *c-fos*, *c-jun*, CREB-1, ATF-2, and p65 were added to the nuclear extracts 30 min prior the binding reaction, and the DNA/nuclear protein complexes were separated on a 4.5% polyacrylamide gel.

Chromatin immunoprecipitation (ChIP) assay

ChIP analysis was performed as described (26). Immunoprecipitated DNA was purified and amplified across the COX-2 promoter region using the 5' primers ⁻¹¹⁹TAAGGGGAGAGGAGGGAAAAAT⁻⁹⁷ and 3' primer ⁺⁶ACAATTGTCGCTAACCGAG⁻¹⁴. PCR products were then resolved in a 1.5% agarose gel.

RT-PCR

Total RNA was isolated from AGS cells using TRIzol Reagent (Invitrogen Life Technologies). The reverse transcription reaction was performed using

2 μ g of total RNA, which was reverse transcribed into cDNA using the oligo dT primer, then the cDNA was amplified for 30 cycles using two oligonucleotide primers derived from a published COX-2 sequence (5'-CAGCACTTACGCATCAGTT-3' and 5'-TCTGGTCAATGGAAGCCTGT-3'), *c-fos* sequence (5'-GAATAACATGGCTGTGCAGCCAAATGCCGAA-3' and 5'-CGTCAGATCAAGGGAAGCCACAGACATCT-3'), *c-jun* sequence (5'-GGAAACGACCTTCTATGACGATGCCCTCAA-3' and 5'-GAACCCCTCCTGTCATCTGTACGTTCTT-3'), and two oligonucleotide primers from a β -actin sequence (5'-TGACGGGGTACCCACACTGTGCCCATCTA-3' and 5'-CTAGAAGCATTTCGGGGGACGATGGAGGG-3'). For COX-2, a PCR cycle consisted of a denaturation step (94°C, 1 min), an annealing step (60°C, 1 min for COX-2 and actin; 58°C, 1 min for *c-fos* and *c-jun*), and an elongation step (72°C, 1.5 min). The total reaction took 35 cycles, which was followed by an additional extension step (72°C, 7 min). For β -actin, PCR cycle was conducted for 30 s at 94°C, 30 s at 65°C, and 1 min at 70°C. The PCR products were subjected to electrophoresis in a 1.5% agarose gel. Quantitative data was obtained using a computing densitometer and ImageQuant software (Molecular Dynamics).

Plasmids

The COX-2 promoter construct pGS459 (-459/+9) was a generous gift from Dr. L. H. Wang (University of Texas, Houston, TX). CRE (CRE-Luc), AP1 (AP1-Luc), and NF- κ B (κ B-Luc) luciferase reporters were from Stratagene. The dominant-negative mutant of ERK2 was from Dr. M. Cobb (South-Western Medical Center, Dallas, TX). p38 (T180A/T182F) was from Dr. J. Han (The Scripps Research Institute, San Diego, CA). JNK (T183A/Y185F) was from Dr. M. Karin (University of California, San Diego, CA). The mutant of TLR2 (P/H) was from Dr. T. Muta (Kyushu University, Fukuoka, Japan) was generated as described previously (27). The mutant of TLR9 (ICD) was from Dr. K. J. Ishii (Food and Drug Administration, Bethesda, MD).

Transient transfection and luciferase activity assay

AGS cells grown to 60% confluence in 12-well plates were transfected with either the human COX-2 pGS-459 or Luc-reporters using SuperFect (Qiagen) according to the manufacturer's instructions. Briefly, reporter DNA (0.3 μ g) and β -galactosidase DNA (0.15 μ g; pRK plasmid containing the β -galactosidase gene driven by the constitutively active SV40 promoter was used to normalize the transfection efficiency) were mixed with 0.45 μ l of SuperFect in 0.4 ml of serum-free RPMI 1640. After 10–15 min of incubation at room temperature, the mixture was applied to the cells. Six hours later, 0.4 ml of RPMI 1640 with 20% FCS was added. Twenty four hours after transfection, the cells were changed into an antibiotic-free medium, and the cells were treated with inhibitors (as indicated) for 30 min and incubated with *H. pylori* for 6 h. Cell extracts were then prepared, and the luciferase and β -galactosidase activities were measured. The luciferase activity was normalized to the β -galactosidase activity.

In experiments using dominant-negative mutants, cells were cotransfected with reporter (0.3 μ g), β -galactosidase (0.15 μ g), and the mutant of TLR2 or TLR9, the dominant-negative mutant of ERK, p38, or JNK, or the empty vector (0.6 μ g). In experiments using the wt plasmids, cells were cotransfected with 0.3 μ g of reporter plasmid, 0.15 μ g of β -galactosidase plasmid, 0.45 μ g of the wt TLR2, 0.6 μ g of the dominant-negative mutant of ERK, p38, or JNK, or the empty vector.

Statistical analysis

Data were analyzed using Student's *t* test. Values of *p* < 0.05 were considered significant.

Results

COX-2-dependent cell invasion and angiogenesis induced by *H. pylori* from cancer patient

Different strains of *H. pylori* from patients with gastric cancer (HC), gastritis (HS), and duodenal ulcer (HD) were analyzed for their capability to induce COX-2 expression and PGE₂ release in the gastric epithelial cells, AGS and MKN45. As in our previous report (14) and as shown in Fig. 1, A and C, HC induced a time-dependent increase of COX-2 expression and PGE₂ release in AGS cells. It was evident at 6 h after *H. pylori* infection and reached maximum at 24 h. A similar result was also observed in MKN45 cells after coculture with HC (Fig. 1, B and D). COX-2 expression and PGE₂ release were seen in

MKN45 cells induced by HD. However, the extent was smaller than that of HC. To evaluate the role of *H. pylori*-induced COX-2 expression in malignant gastric cell invasion, a Matrigel assay was performed. The invasion of AGS cells was induced by HC, but not HS and HD, and HC-induced AGS cell invasion was inhibited by two COX-2 inhibitors, NS-398 and celecoxib (Fig. 1E). To investigate the mechanism of COX-2-dependent gastric tumor cell invasion, exogenous PGE₂ was added. PGE₂ (50 nM) enhanced cell invasion and reversed the inhibition of NS-398 (Fig. 1E). Similar results were seen in MKN45 cells cocultured with HC (Fig. 1F). HD, which induced COX-2 expression and PGE₂ release in the MKN45 cells, also elicited MKN45 cell invasion (Fig. 1F).

To investigate the in vitro angiogenesis caused by *H. pylori*, Matrigel tube formation assay was performed in HUVECs. The tube structures were significantly formulated when HUVECs were treated with the condition media collected from AGS or MKN45 cells cocultured with HC (Fig. 1G). Condition media collected from HS also induced a smaller extent of tube formation. When pretreated by NS-398, the tube formation induced by HC was incomplete and the number was reduced (Fig. 1G). These results demonstrated that PGE₂ release to the media due to COX-2 expression elicited tube formation in HUVECs.

H. pylori acts through TLR2/TLR9-mediated COX-2 to induce tumor cell invasion and tube formation

Because TLR2 and TLR9 were demonstrated to be involved in the *H. pylori*-induced COX-2 expression (14), their role in the *H. pylori*-induced cell invasion was examined. The AGS cell line over-expressing COX-2, AGS/COX-2, was established and used to examine the role of COX-2 in cell invasion and angiogenesis. As shown in Fig. 2A, this stable clone had an abundant expression of COX-2 and showed high invasiveness comparing to the mock, AGS/pcDNA3. Neutralizing Abs to TLR2 and TLR9 attenuated the *H. pylori*-induced cell invasion, but they did not affect the invasiveness of AGS/COX-2 cells (Fig. 2A). These results demonstrated that *H. pylori* induced cell invasion via TLR2/TLR9-mediated COX-2 expression in AGS cells.

Condition media collected from AGS/COX-2 induced a significant tube formation (Fig. 2B). Neutralizing Abs to TLR2 and TLR9 attenuated the *H. pylori*-induced tube formation. However, they did not affect the tube formation induced by AGS/COX-2 cells (Fig. 2B). These results demonstrated that TLR2/TLR9-dependent COX-2 expression and PGE₂ release mediated by *H. pylori*-induced tube formation in vitro.

Bindings of CREB-1, ATF-2, *c-jun*, and *c-fos* to the CRE and AP-1 elements on the COX-2 promoter

The CRE site (-59/-53) on the human COX-2 promoter is adjacent to the upstream AP-1 site (-67/-61) (Fig. 3A). DAPA experiments were performed to analyze the transcription factor binding to the respective CRE and AP-1 elements after *H. pylori* infection. A time-dependent increase in the bindings of CREB1, ATF-2, *c-jun*, and *c-fos* to the CRE plus AP1 site were found. The p65, which is a component of NF- κ B, did not bind to the CRE plus AP1 site (Fig. 3B). Probes containing consensus sequences of CRE plus AP1, with CRE mutation (CRE mut) or AP1 mutation (AP1 mut) were used to clarify the component binding to the CRE and AP1 sites, respectively. Increases in the binding of CREB-1, ATF-2, and *c-jun* to the CRE site (Fig. 3C, compare lanes 6 and 5), and *c-fos*, *c-jun*, and ATF-2 to the AP1 site (Fig. 3C, compare lanes 4 and 3) after *H. pylori* infection were identified.

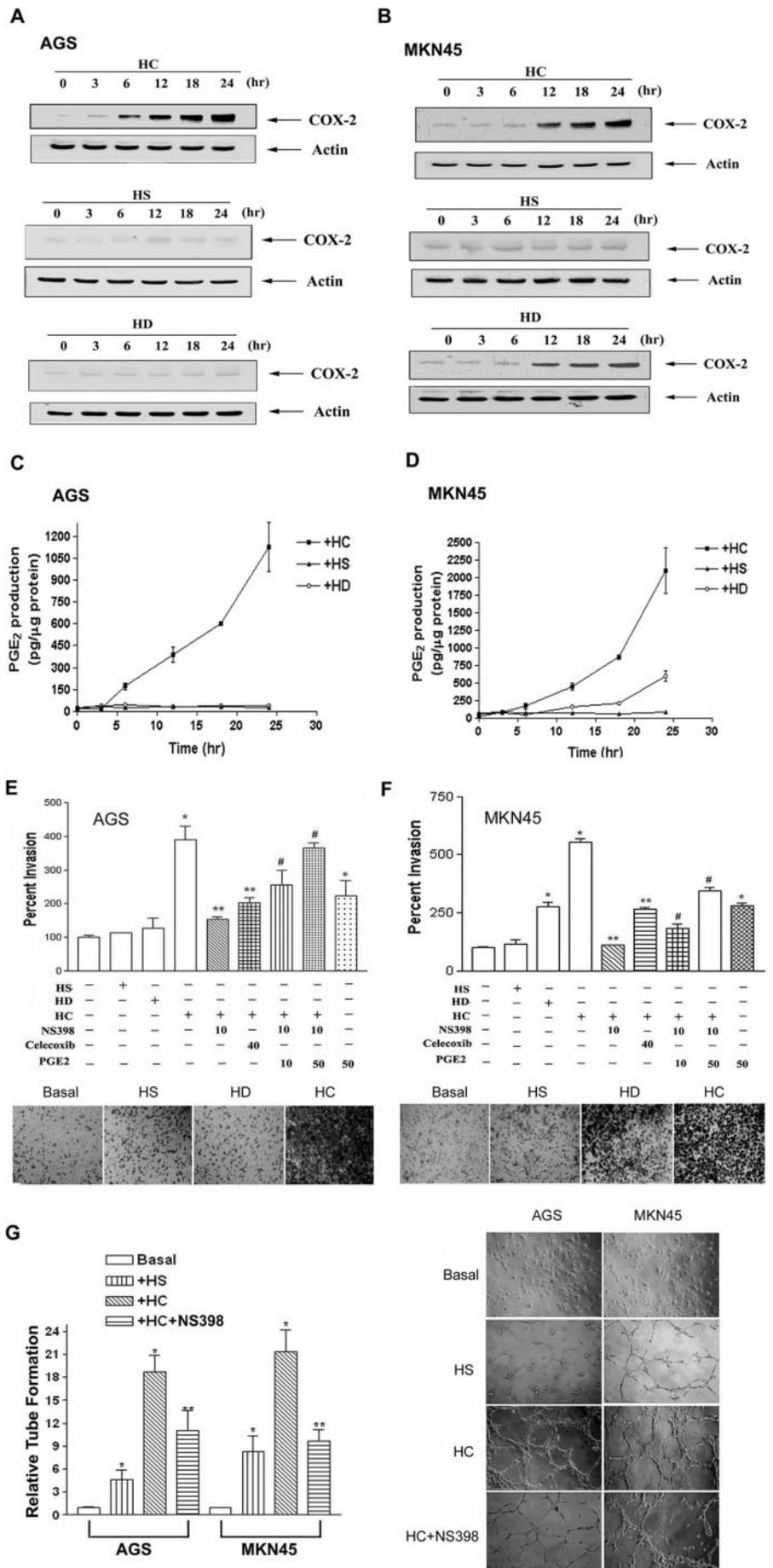


FIGURE 1. COX-2-dependent tumor cell invasion and angiogenesis induced by *H. pylori* isolates from patients. AGS (A) or MKN45 (B) cells were cocultured with HC, HS, or HD at a bacterium to cell ratio of 150:1 for the indicated time. Whole cell lysates were prepared and subjected to Western blot using Ab specific to COX-2 or actin. PGE₂ releases in the media collected from AGS (C) or MKN45 (D) cells were measured using ELISA. The PGE₂ production was normalized to the total protein level. AGS (E) or MKN45 (F) cells were treated with 50 nM PGE₂ or cocultured with HS, HD, or HC for 48 h followed by pretreatment with 10 μM NS-398, 40 μM celecoxib, 10 nM PGE₂, or 50 nM PGE₂ on Transwell inserts coated with Matrigel. The percent invasion was measured by comparing with the basal (*, $p < 0.05$ as compared with the basal; **, $p < 0.05$ as compared with HC; #, $p < 0.05$ as compared with HC in the presence of NS-398). The images of invasive cells stained with crystal violet were shown in the lower panels. G, Photomicrographs depicted a random field of view and showed the alignment of HUVECs treated with condition media collected from gastric epithelial cells rendered with different treatments. “Basal” means the media collected from cells without any treatment. “HS” means the media collected from cells cocultured with HS for 16 h. “HC” means the media collected from cells cocultured with HC for 16 h. “HC + NS398” means the media collected from cells cocultured with HC for 16 h followed by pretreatment with NS398 for 30 min (right panel). Quantification of the extent of tube formation was shown as relative to the control (left panel). Data are presented as means ± SE of three independent experiments with six replicates per condition (*, $p < 0.05$ vs basal; **, $p < 0.05$ as compared with HC alone).

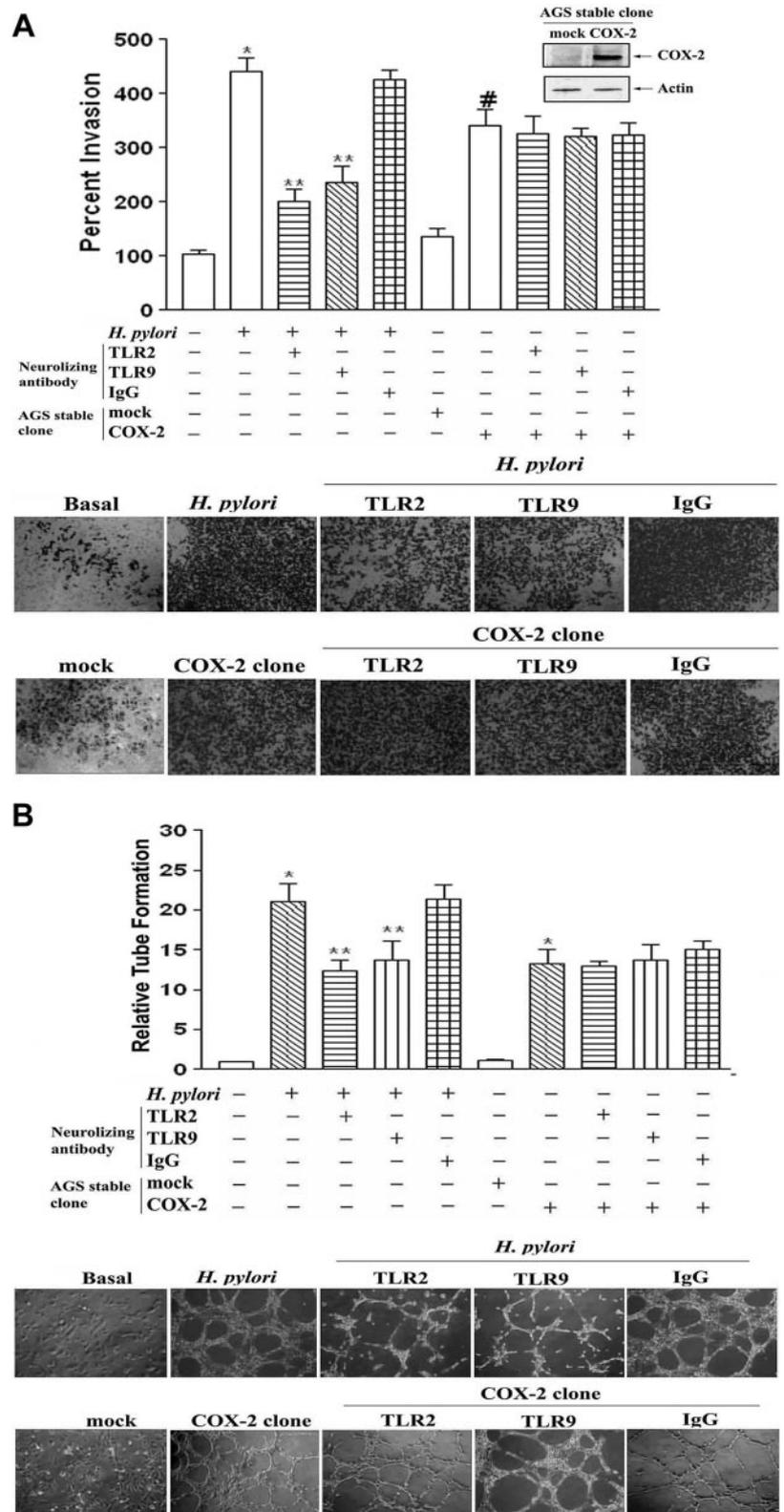


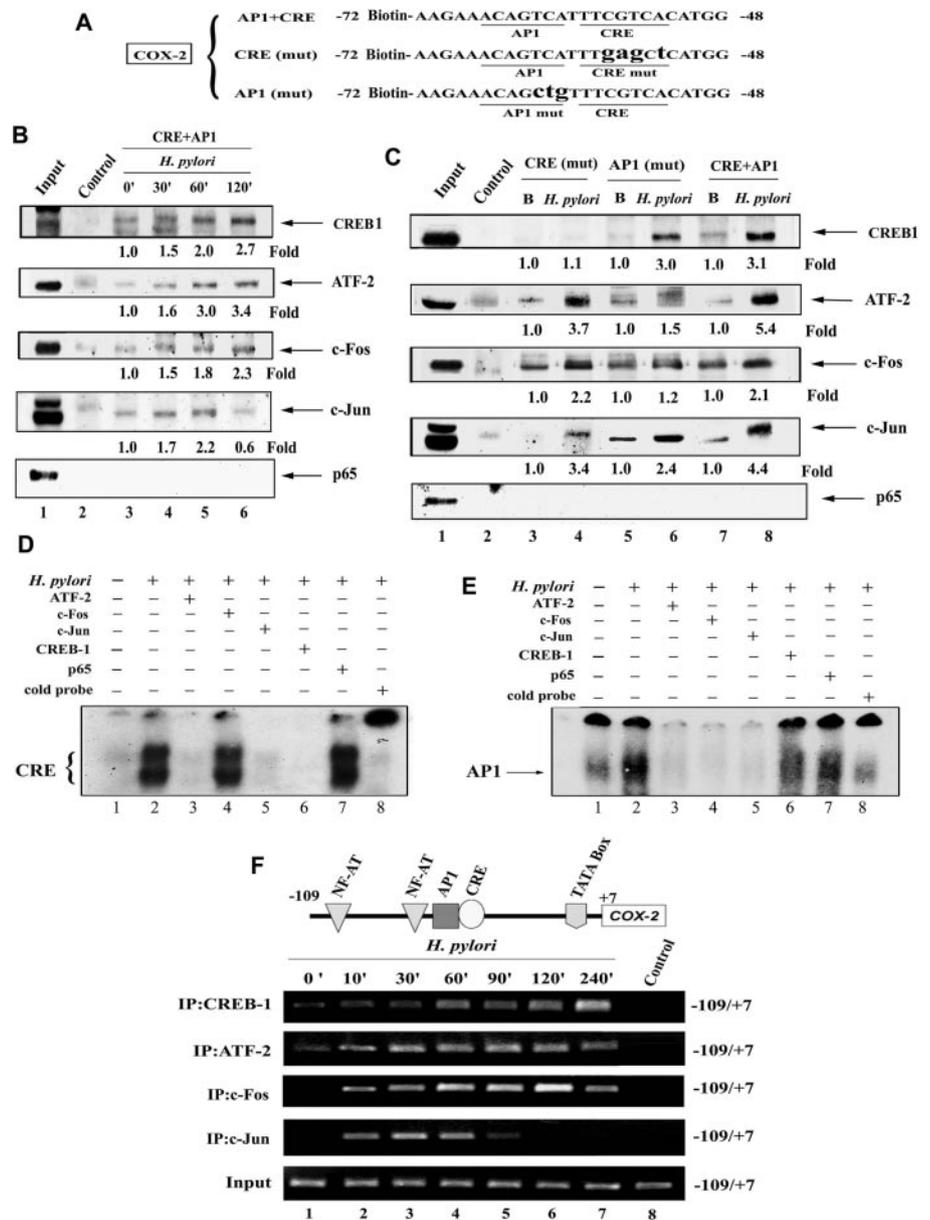
FIGURE 2. *H. pylori* acts through TLR2- and TLR9-mediated COX-2 to induce tumor cell invasion and angiogenesis. **A**, Mock (AGS/pcDNA3) and AGS cells overexpressing COX-2 (AGS/COX-2) were preincubated with rabbit anti-human TLR2 or goat anti-human TLR9 Ab, or IgG (Santa Cruz Biotechnology) for 3 h and cocultured with or without *H. pylori* (MOI, 150) for 48 h, then cell invasions were measured (**, $p < 0.05$ as compared with *H. pylori* alone; #, $p < 0.05$ as compared with mock). The images of invasive cells stained with crystal violet were shown in the lower panel. **B**, AGS/pcDNA3 and AGS/COX-2 cells were preincubated with anti-TLR2 or anti-TLR9 Ab, or IgG for 3 h, then cocultured with or without *H. pylori* (MOI, 150) for 16 h. Quantification of the extent of tube formation was shown as relative to the basal. Data are presented as means \pm SE of three independent experiments with six replicates per condition (*, $p < 0.05$ as compared with basal or mock; **, $p < 0.05$ as compared with *H. pylori* alone).

To further confirm the results from DAPA, supershift experiments were performed. Anti-ATF-2, *c-jun*, CREB1, but not *c-fos* and p65, Abs blocked the CRE-specific DNA-protein complex formation using probe with CRE consensus sequences (Fig. 3D, lanes 3–7). Anti-ATF-2, *c-fos*, *c-jun*, not CREB1 and p65, Abs blocked the AP1-specific DNA-protein complex formation using probe with AP1 consensus sequences (Fig. 3E, lanes 3–7). These data

confirmed the bindings of CREB-1, ATF-2, and *c-jun* to the CRE site, and *c-fos*, *c-jun*, and ATF-2 to the AP1 site.

The in vivo recruitments of CREB1, ATF-2, *c-jun*, and *c-fos* to the COX-2 promoter induced by *H. pylori* were demonstrated by ChIP assay. As shown in Fig. 3F, time-dependent increases in the binding of these transcription factors to the promoter region –109/+7 containing CRE and AP1 sites were found.

FIGURE 3. Time-dependent increase of *H. pylori*-induced CREB-1, ATF-2, c-Fos, and c-Jun bindings to the COX-2 promoter in gastric epithelial cells. **A**, Schematic illustration of the consensus sequences of CRE plus AP-1 sites on the human COX-2 promoter labeled with biotin. The underlined shows the region corresponding to CRE and AP1 consensus sequences. Bases mutated in the CRE or AP1 site are indicated by bold letters. **B** and **C**, *H. pylori*-induced in vitro bindings of CREB-1, ATF-2, c-Fos, and c-Jun to the COX-2 promoter by DAPA. AGS cells were cocultured with *H. pylori* for 30, 60, and 120 min (**B**), or 60 min (**C**), then nuclear extracts were prepared and incubated with the biotinylated AP1 plus CRE probe (**B**), the biotinylated CRE (mut) or AP1 (mut), or CRE plus AP1 probe (**C**), or without probe (Control). The resulting complexes were precipitated by streptavidin-agarose beads. CREB1, ATF-2, c-Fos, c-Jun, or p65 in the complexes, and nuclear extracts (Input) were detected using Western blot. **D** and **E**, *H. pylori*-induced CREB-1, ATF-2, c-Fos, and c-Jun binding to the COX-2 promoter by supershift assay. AGS cells were cocultured with *H. pylori* for 60 min, then nuclear extracts were prepared, and DNA-protein complex formation was measured. The supershift assays were performed using 2 μ g of the indicated Abs, then nuclear extracts were incubated with the probe containing either CRE (**D**) or AP1 (**E**) consensus sequences. **F**, *H. pylori*-induced in vivo bindings of CREB-1, ATF-2, c-Fos, and c-Jun to the COX-2 promoter by ChIP assay. Schematic illustration of various transcription activator binding sites on the proximal region of human COX-2 promoter (-109/+7) was shown in the upper panel. Cells were cocultured with *H. pylori* for the indicated time, then ChIP assays were performed using anti-CREB-1, anti-ATF-2, anti-c-jun, anti-c-fos Ab, or without Ab (Control) to assay the precipitated COX-2 promoter -109/+7 region. One percent of total chromatin was assayed to verify equal loading (Input).



Regulation of *H. pylori*-induced c-fos and c-jun mRNA expressions, and CREB-1 and ATF-2 activations by MAPKs

Because *c-fos* and/or *c-jun* were demonstrated to be the components binding to the CRE and AP1 sites, their transcriptional regulations were examined. Time-dependent increases in the *c-fos* and *c-jun* mRNA expressions in AGS cells infected by *H. pylori* were seen (Fig. 4A). Because the expressions of *c-fos* and *c-jun* were regulated by MAPKs (28), the roles of ERK1/2, p38, and JNK in this event were examined. Similar to our previous findings (28), PD98059 (50 μ M) blocked TNF- α -induced ERK1/2 activation without having any effect on the p38 and JNK activations, and SB 203580 (30 μ M) caused complete inhibition on p38 activation without affecting ERK1/2 and JNK activations. SP600125 inhibited JNK1/2 activation in a dose-dependent manner without any effect on ERK1/2 and p38 activations (data not shown). PD98059 and SB203580 were shown to inhibit the *H. pylori*-induced *c-fos* mRNA expression, whereas SB203580 and SP600125 inhibited the *c-jun* mRNA expression (Fig. 4B, lanes 3 and 4; lanes 4–6). Taken together, these results indicated the involvement of ERK and p38, not

JNK, in the regulation of *c-fos* mRNA expression and p38 and JNK, not ERK, in the *c-jun* mRNA expression.

Activations of CREB-1 and ATF-2 were examined by their phosphorylation. *H. pylori*-induced phosphorylation of CREB-1 in the nuclear extracts was seen after coculture with *H. pylori* for 10 min and sustaining for 120 min (Fig. 4C, lanes 2–6). Phosphorylation of ATF-2 in the nuclear extracts reached maximum at 10 min and declined at 60 min (Fig. 4E, lanes 2–4). The roles of MAPKs in the phosphorylations of CREB-1 and ATF-2 were examined. ERK and p38, not JNK, were found to regulate the phosphorylation of CREB-1. However, all three MAPKs were shown to regulate the ATF-2 phosphorylation (Fig. 4, D and F).

Involvements of TLR2/9, ERK, p38, and JNK in *H. pylori*-mediated COX-2 promoter activity via activations of CRE and AP-1

Because TLR2/9 and CRE and AP-1 sites on the COX-2 promoter were demonstrated to be involved in the *H. pylori*-induced COX-2 expression, *H. pylori*-induced CRE and AP-1 activation through TLR2/9 was examined. As shown in Fig. 5, A

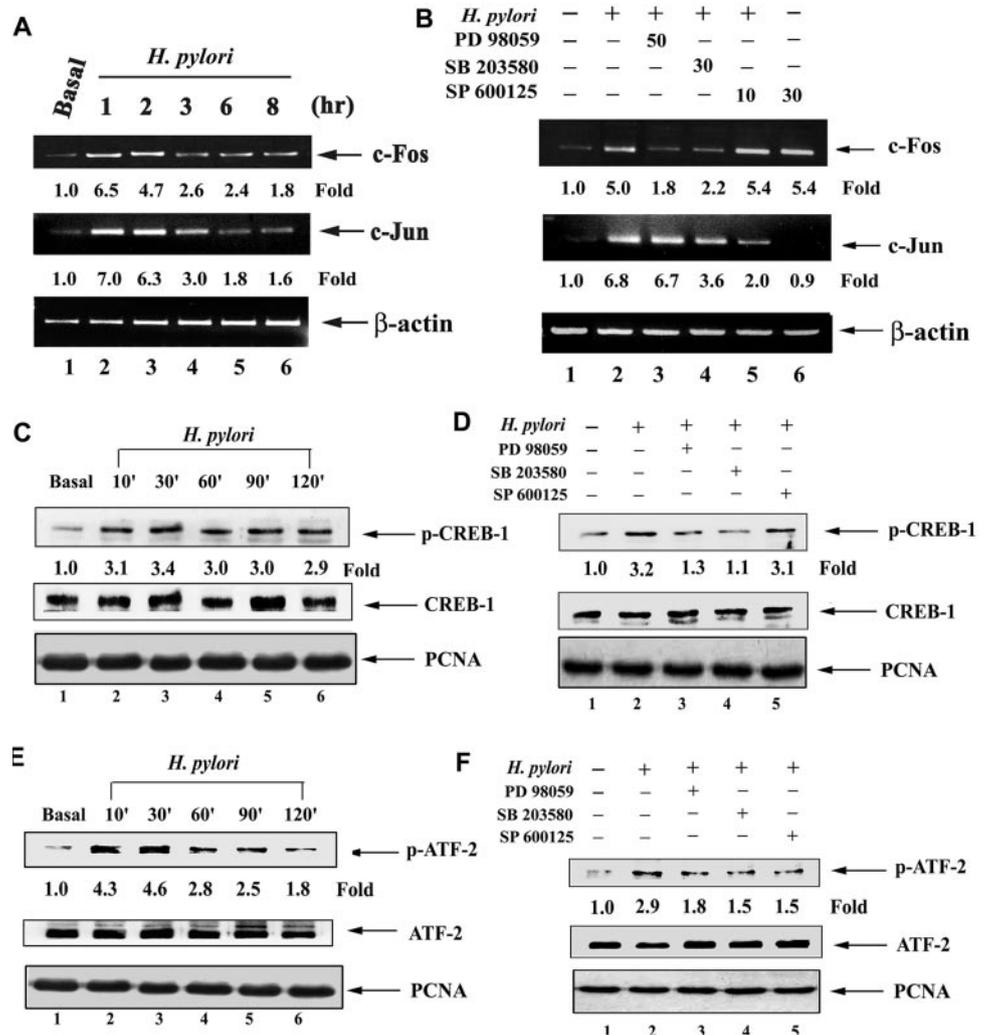


FIGURE 4. The expressions of *c-fos* and *c-jun* mRNA and phosphorylations of CREB-1 and ATF-2 induced by *H. pylori* and effects of MAPKs inhibitors. AGS cells were cocultured with *H. pylori* (MOI, 150) for the indicated time (A) or with *H. pylori* (MOI, 150) for 1 h followed by the pretreatment with PD98059 (50 μ M), SB203580 (30 μ M), or SP600125 (10 or 30 μ M) for 30 min (B). Total RNA (2 μ g) was used for RT-PCR. C-F, AGS cells were cocultured with *H. pylori* (MOI, 150) for the indicated time or with *H. pylori* (MOI, 150) for 30 min followed by the pretreatment with PD98059 (50 μ M), SB203580 (30 μ M), or SP600125 (30 μ M) for 30 min. The nuclear extracts were prepared and subjected to Western blot using Abs specific for p-CREB1 and CREB1 (C and D) or p-ATF2 and ATF-2 (E and F).

and B, *H. pylori*-induced CRE and AP-1 luciferase activities were inhibited by the TLR2 and TLR9 mutants, suggesting the involvements of these two TLRs. Because the binding components of CRE and AP-1 sites and their regulations by MAPKs were demonstrated, the roles of MAPKs in regulating the *H. pylori*-induced CRE and AP-1 luciferase activities were further confirmed using the dominant-negative p38, ERK2, and JNK mutants (Fig. 5, C and D).

Activation of PKC/c-Src/NF- κ B by *H. pylori* to induce COX-2 expression was recently found by this lab (14). To examine the possible involvement of MAPKs in regulating NF- κ B activity, *H. pylori*-induced NF- κ B-luc activity was examined in the presence of the dominant-negative mutants of MAPKs. In Fig. 5E, the induction of NF- κ B activity by *H. pylori* was not affected by these dominant-negative mutants, and the *H. pylori*-induced I κ B α degradation was not reversed by either PD98059, SB308520, or SP600125 (Fig. 5F, lanes 3–6). These results suggested that MAPKs do not act through the NF- κ B site to induce COX-2 expression in the *H. pylori*-infected AGS cells.

Because MAPKs-regulated CRE and AP-1 sites were found to be involved in the *H. pylori*-induced COX-2 expression, the role of MAPKs in the *H. pylori*-induced COX-2 promoter activity was examined. As shown in Fig. 5G, the COX-2 promoter activity was stimulated by HC but not HS, which was in line with the results that HC, but not HS, could induce COX-2 expression in AGS cells (Ref. 14; Fig. 1A). *H. pylori*-induced COX-2 promoter activity was

really inhibited by the MAPKs inhibitors and their dominant-negative mutants (Fig. 5, H and I). The *H. pylori*-induced COX-2 mRNA and protein were also attenuated by the MAPKs inhibitors (Fig. 5, J and K), confirming the involvements of ERK, p38, and JNK in the *H. pylori*-induced CRE and AP1 activations and the COX-2 expression.

Discussion

An increase in COX-2 expression has been implicated in the genesis of a variety of human cancers, including colorectal, gastric, pancreatic, esophageal, brain, and lung cancers (29). COX-2 may play a role as survival factor in protecting cells from apoptosis (30). Furthermore, functions like the encouragement of cell proliferation (31), the induction of tumor cell invasion (32, 33), the stimulation of angiogenesis (11), and the inhibition of immunosurveillance (34) have also been described. SC-236, a selective COX-2 inhibitor, was effective in reducing angiogenesis driven by the basic fibroblast growth factor in a Matrigel model (35). The expression of COX-2 in the newly formed blood vessels within tumors grown in animals was observed, whereas the quiescent vascula expressed only COX-1, indicating the involvement of COX-2-derived PGs in tumor angiogenesis (35). We demonstrated that *H. pylori* isolated from gastric cancer patients induced the COX-2 expression and PGE₂ release, and they also increased the angiogenesis and cell invasion in the AGS and MKN45 cells. The highly specific COX-2 inhibitors, NS-398 and celecoxib, were shown to

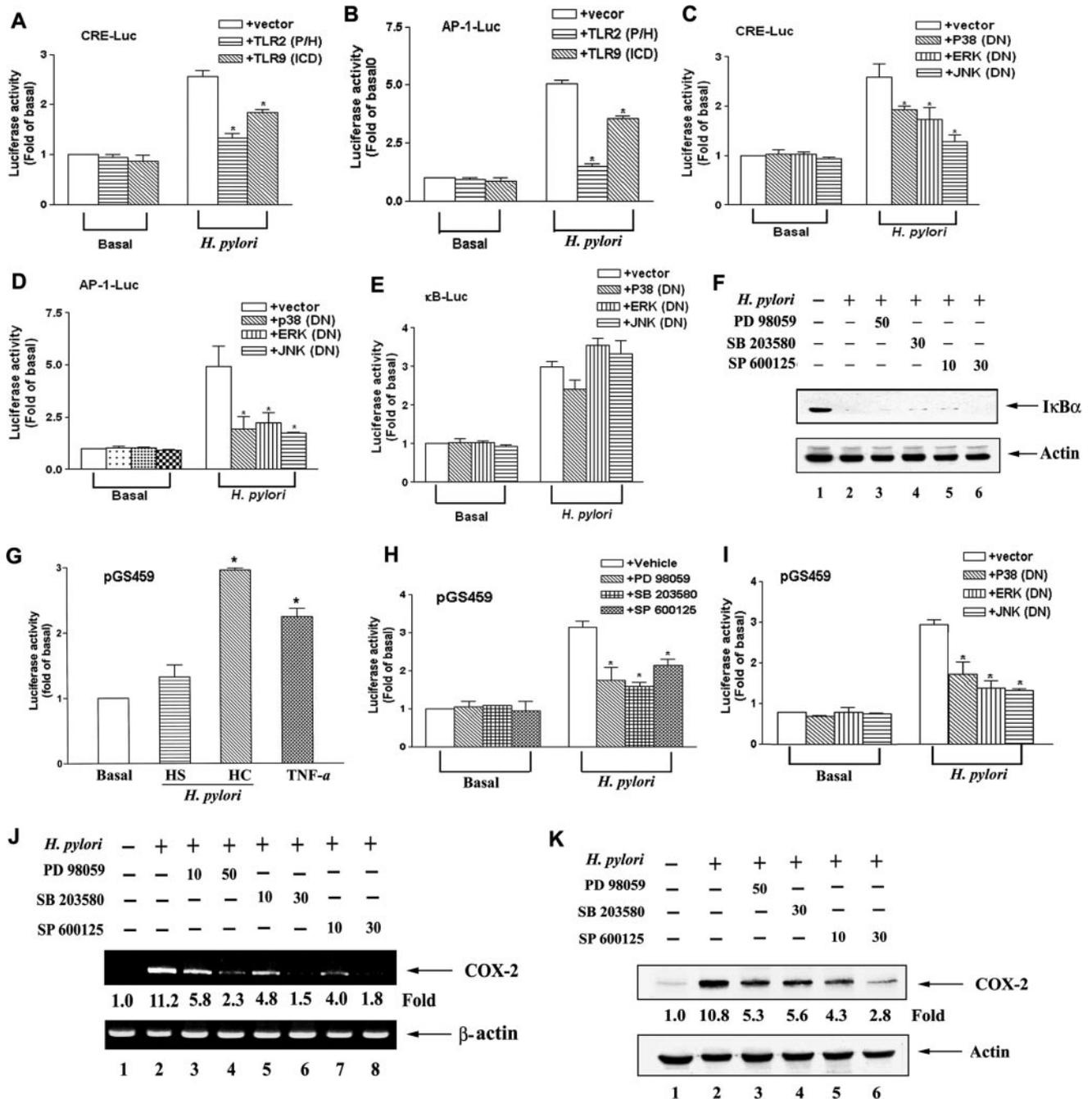


FIGURE 5. Roles of TLR2, TLR9, and MAPKs in *H. pylori*-induced CRE, AP1, NF- κ B, or COX-2 promoter activity and COX-2 expression. AGS cells were cotransfected with either CRE-Luc or AP1-Luc and the mutant of TLR2 or TLR9 (A and B) or the dominant-negative mutant of MAPKs (C and D), then cocultured with *H. pylori* (MOI, 150) for 6 h. Cells were cotransfected with NF- κ B-Luc and the dominant-negative mutant of MAPKs, then cocultured with *H. pylori* (MOI, 150) for 6 h (E). Luciferase activity was measured. F, Cells were cocultured with *H. pylori* (MOI, 150) for 30 min followed by the pretreatment with PD98059 (50 μ M), SB203580 (30 μ M), or SP600125 (10 or 30 μ M) for 30 min. Whole cell lysates were prepared and subjected to Western blot using Abs specific to I κ B α or actin. G, AGS cells were transfected with pGS459, then cocultured with *H. pylori* HS or HC (MOI, 150), or TNF- α (10 ng/ml) for 6 h. H and I, AGS cells were transfected with pGS459 before pretreatment with PD98059 (50 μ M), SB203580 (30 μ M), or SP600125 (30 μ M) for 30 min (H), or cotransfected with the dominant-negative mutants of MAPKs or the respective empty vector (I), then cocultured with *H. pylori* (MOI, 150) for 6 h. Luciferase activity was measured. The results were normalized to β -galactosidase activity and expressed as the mean \pm SE of three independent experiments performed in triplicate (*, $p < 0.05$ as compared with *H. pylori* alone). J and K, cells were pretreated with PD98059 (10 or 50 μ M), SB203580 (10 or 30 μ M), or SP600125 (10 or 30 μ M) for 30 min before coculture with *H. pylori* (MOI, 150) for 3 h for RT-PCR (J) or 16 h for Western blot (K).

inhibit these phenomena. The introduction of exogenous PGE₂ reversed the inhibitory effect of NS-398. Similar positive correlation between PGs productions and tumor invasiveness was observed in the lung cells (32). Cell line transfected with COX-2 (AGS/COX-2) also exhibited high invasiveness and angiogenesis. Our

previous study has shown that neutralizing Abs for TLR2 and TLR9 blocked *H. pylori*-induced COX-2 expression (14). In this study, they also attenuated the *H. pylori*-induced but not the AGS/COX-2 cells-induced invasion and angiogenesis, confirming the involvement of TLR2 and TLR9 in the effect of *H. pylori*. These

results were in line with the recent studies of *H. pylori* activating innate immunity and NF- κ B signaling via TLR2 but not TLR4 (36–38), and finding of TLR9 expression on the gastric epithelial cell surface (39). Moreover, TLR2 and TLR9 agonists were found to promote an angiogenic switch (40). These findings together revealed a novel function of TLR2 and TLR9 in *H. pylori*-induced COX-2 expression, cell invasion, and angiogenesis. However, the addition of both anti-TLR2 and TLR9 Abs to the culture could not completely block cell invasion and tube formation (data not shown), indicating that other TLR receptors or alternative mechanisms may be functional.

The effectiveness of NS-398 or celecoxib on inhibiting cell invasion and angiogenesis of AGS and MKN45 gastric cancer cell lines points to a promising therapeutic target for gastric cancer prevention and treatment, as exemplified by the great success of COX-2 inhibitor in the colon cancer prevention and treatment (41). Therefore, COX-2 and its metabolites are the necessary “angiogenic” and “invasive/permmissive” factors and are sufficient to induce tumor invasiveness and angiogenesis. Recently, the use of nonsteroidal anti-inflammatory drugs is also reported to be associated with a decreased risk of gastric cancer in a systematic review and metaanalysis of epidemiological evidence (42). Intriguingly, the protective effect of nonsteroidal anti-inflammatory drugs was observed only in individuals who were *H. pylori* IgG-positive but not in noninfected subjects (43).

The mechanism that *H. pylori* induced COX-2 expression at transcriptional level was further examined. Our recent data demonstrated the involvements of CRE (–59/–53), NF-IL6, and NF- κ B sites in the *H. pylori*-induced COX-2 expression (14). Juttner et al. (44) also reported the involvement of CRE/E box (–56/–48) in *H. pylori*-induced COX-2 expression. However, we further verified and investigated the AP-1 element (–67/–61). The transcription factors of AP-1 family (Fos, Jun, and ATF) and CREB/ATF family (ATF and CREB) can form either homo- or heterodimer. Jun-Jun and Jun-Fos dimers preferentially bind to the AP-1 site, whereas Jun-ATF prefers binding to the CRE sequence (45). Using DAPA and supershift assays, the respective bindings of CREB-1, ATF-2, and *c-jun* to the CRE site, and *c-fos*, *c-jun*, and ATF-2 to the AP1 site after *H. pylori* coculture were demonstrated. In addition, ChIP assays demonstrated the in vivo recruitments of CREB-1, ATF-2, *c-jun*, and *c-fos* to the proximal sites of COX-2 promoter (–109/+7). We are the first to clearly verify the respective binding components of CRE and AP-1 sites on the COX-2 promoter both in vivo and in vitro. Dannenberg and colleagues and others claimed the binding of AP-1 components to the CRE site using oligonucleotide consensus sequences containing both CRE and AP-1 sites (21, 22, 46). In their experiments, the individual role of CRE and AP-1 could not be clearly differentiated because they claimed the bindings of *c-fos*, *c-jun*, and ATF-2 (AP-1 components) to the CRE site (21). However, Bowden and colleagues (47) found the bindings of CREB and ATF-1 (CREB components) to the CRE site using similar probes. We and Flamand and colleagues (18) used oligonucleotides containing respective AP-1 site- and CRE site-specific consensus binding sequences. Flamand and colleagues also demonstrated the involvements of CRE and AP-1 sites in the COX-2 promoter activation in human herpes virus 6-infected monocytes. They found the binding of CREB-1 to the CRE site and *c-jun* to the AP-1 site (18). Therefore, respective binding components of CRE and AP-1 sites on the COX-2 promoter were explored. Our unpublished data demonstrated the specific binding of CREB/ATF-2 to the CRE site (–53/–45), and *c-fos/c-jun* to the AP-1

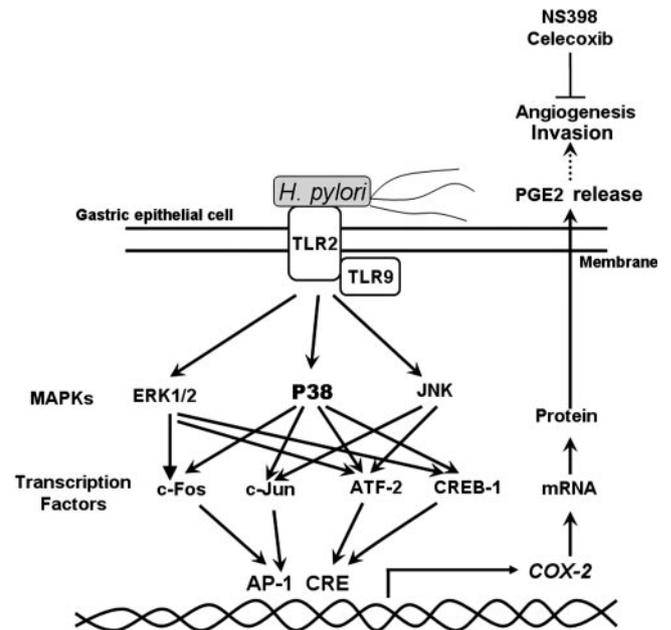


FIGURE 6. Schematic representation of the signaling pathways involved in *H. pylori*-induced COX-2 expression in AGS epithelial cells. *H. pylori* acts through TLR2 and TLR9 to activate MAPKs, especially p38, leading to the phosphorylations of ATF-2 and CREB1 as well as increases in *c-fos* and *c-jun* expressions, which, in turn, result in the activation of CRE and AP1 elements on the human COX-2 promoter and the initiation of COX-2 expression. This COX-2 induction increases the cell invasion and angiogenesis of gastric cancer and is attenuated by the COX-2 inhibitors.

site (–936/–930) on the cyclin D1 promoter after *H. pylori* infection. The additional bindings of *c-jun* to the CRE site and ATF-2 to the AP1 site in the present study might be due to the adjacency of these two elements on the COX-2 promoter, as they are far apart on the cyclin D1 promoter.

Present studies explored that *H. pylori* induced activations of CRE and AP-1 through MAPKs pathways. As lung A549 cells expressing ICAM-1 and releasing IL-8 through MAPKs-induced AP-1 activation (28, 48), ERK and p38 regulate *c-fos* mRNA expression, and JNK and p38 regulate *c-jun* mRNA expression in the present AGS cells. Activations of ERK and *c-fos* expression by *H. pylori* have also been noted (49). CREB-dependent transcription is regulated through the phosphorylation of serine 133, which was first found to be mediated by PKA (50). Our finding provided evidence for an alternative mechanism whereby CREB was phosphorylated by MAPKs as found by Yu et al. (51). ATF-2 has been reported to be activated by JNK and p38 (52, 53). In this study, we showed that ATF-2 was activated by all three MAPKs. Therefore, the binding components of CRE and AP-1 sites are differentially regulated by the MAPKs. Intriguingly, p38 could regulate all these transcription factors.

In summary, we provided a mechanistic view of COX-2 in the *H. pylori*-mediated carcinogenesis. *H. pylori* was shown to act through TLR2 and TLR9 to activate the MAPKs (ERK1/2, p38, JNK) and their downstream transcription factors (CREB-1, ATF-2, *c-fos*, and *c-jun*), resulting in the activations of CRE and AP-1 on the COX-2 promoter. These intracellular networks drive the COX-2-dependent PGE₂ release, and they may contribute to the carcinogenesis and metastasis in patients colonized with these strains through the enhancement of tumor cell invasion and angiogenesis. A schematic diagram exhibiting the

comprehensive intracellular networks in gastric cancer cells is displayed in Fig. 6, and the application of the selective COX-2 inhibitor may provide an alternative therapy to reduce the development of gastric cancer.

Disclosures

The authors have no financial conflict of interest

References

- Montecucco, C., and R. Rappuoli. 2001. Living dangerously: how *Helicobacter pylori* survives in the human stomach. *Nat. Rev. Mol. Cell Biol.* 2: 457–466.
- Peek, R. M., Jr., and M. J. Blaser. 2002. *Helicobacter pylori* and gastrointestinal tract adenocarcinomas. *Nat. Rev. Cancer* 2: 28–37.
- Uemura, N., T. Mukai, S. Okamoto, S. Yamaguchi, H. Mashiba, K. Taniyama, N. Sasaki, K. Haruma, K. Sumii, and G. Kajiyama. 1997. Effect of *Helicobacter pylori* eradication on subsequent development of cancer after endoscopic resection of early gastric cancer. *Cancer Epidemiol. Biomarkers Prev.* 6: 639–642.
- Fu, S., K. S. Ramanujam, A. Wong, G. T. Fantry, C. B. Drachenberg, S. P. James, S. J. Meltzer, and K. T. Wilson. 1999. Increased expression and cellular localization of inducible nitric oxide synthase and cyclooxygenase 2 in *Helicobacter pylori* gastritis. *Gastroenterology* 116: 1319–1329.
- Lim, H. Y., H. J. Joo, J. H. Choi, J. W. Yi, M. S. Yang, D. Y. Cho, H. S. Kim, D. K. Nam, K. B. Lee, and H. C. Kim. 2000. Increased expression of cyclooxygenase-2 protein in human gastric carcinoma. *Clin. Cancer Res.* 6: 519–525.
- Wambura, C., N. Aoyama, D. Shirasaka, T. Sakai, T. Ikemura, M. Sakashita, S. Maekawa, K. Kuroda, T. Inoue, S. Ebara, et al. 2002. Effect of *Helicobacter pylori*-induced cyclooxygenase-2 on gastric epithelial cell kinetics: implication for gastric carcinogenesis. *Helicobacter* 7: 129–138.
- Romano, M., V. Ricci, A. Memoli, C. Tuccillo, P. A. Di, P. Sommi, A. M. Acquaviva, B. C. Del Vecchio, C. B. Bruni, and R. Zarrilli. 1998. *Helicobacter pylori* up-regulates cyclooxygenase-2 mRNA expression and prostaglandin E₂ synthesis in MKN 28 gastric mucosal cells in vitro. *J. Biol. Chem.* 273: 28560–28563.
- Folkman, J. 1995. Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat. Med.* 1: 27–31.
- Kitadai, Y., A. Sasaki, M. Ito, S. Tanaka, N. Oue, W. Yasui, M. Aihara, K. Imagawa, K. Haruma, and K. Chayama. 2003. *Helicobacter pylori* infection influences expression of genes related to angiogenesis and invasion in human gastric carcinoma cells. *Biochem. Biophys. Res. Commun.* 311: 809–814.
- Strowski, M. Z., T. Cramer, G. Schafer, S. Juttner, A. Walduck, E. Schipani, W. Kemmer, S. Wessler, C. Wunder, M. Weber, et al. 2004. *Helicobacter pylori* stimulates host vascular endothelial growth factor-A (vegf-A) gene expression via MEK/ERK-dependent activation of Sp1 and Sp3. *FASEB J.* 18: 218–220.
- Tsujii, M., S. Kawano, S. Tsujii, H. Sawaoka, M. Hori, and R. N. DuBois. 1998. Cyclooxygenase regulates angiogenesis induced by colon cancer cells. *Cell* 93: 705–716.
- Cario, E., I. M. Rosenberg, S. L. Brandwein, P. L. Beck, H. C. Reinecker, and D. K. Podolsky. 2000. Lipopolysaccharide activates distinct signaling pathways in intestinal epithelial cell lines expressing Toll-like receptors. *J. Immunol.* 164: 966–972.
- Barton, G. M., and R. Medzhitov. 2003. Toll-like receptor signaling pathways. *Science* 300: 1524–1525.
- Chang, Y. J., M. S. Wu, J. T. Lin, B. S. Sheu, T. Muta, H. Inoue, and C. C. Chen. 2004. Induction of cyclooxygenase-2 overexpression in human gastric epithelial cells by *Helicobacter pylori* involves TLR2/TLR9 and c-Src-dependent nuclear factor- κ B activation. *Mol. Pharmacol.* 66: 1465–1477.
- Crawford, H. C., U. S. Krishna, D. A. Israel, L. M. Matrisian, M. K. Washington, and R. M. Peek, Jr. 2003. *Helicobacter pylori* strain-selective induction of matrix metalloproteinase-7 in vitro and within gastric mucosa. *Gastroenterology* 125: 1125–1136.
- Keates, S., A. C. Keates, M. Warny, R. M. Peek, Jr., P. G. Murray, and C. P. Kelly. 1999. Differential activation of mitogen-activated protein kinases in AGS gastric epithelial cells by cag⁺ and cag⁻ *Helicobacter pylori*. *J. Immunol.* 163: 5552–5559.
- Iniguez, M. A., S. Martinez-Martinez, C. Punzon, J. M. Redondo, and M. Fresno. 2000. An essential role of the nuclear factor of activated T cells in the regulation of the expression of the cyclooxygenase-2 gene in human T lymphocytes. *J. Biol. Chem.* 275: 23627–23635.
- Janelle, M. E., A. Gravel, J. Gosselin, M. J. Tremblay, and L. Flamand. 2002. Activation of monocyte cyclooxygenase-2 gene expression by human herpesvirus 6: role for cyclic AMP-responsive element-binding protein and activator protein-1. *J. Biol. Chem.* 277: 30665–30674.
- Ogasawara, A., T. Arakawa, T. Kaneda, T. Takuma, T. Sato, H. Kaneko, M. Kumegawa, and Y. Hakeda. 2001. Fluid shear stress-induced cyclooxygenase-2 expression is mediated by C/EBP β , cAMP-response element-binding protein, and AP-1 in osteoblastic MC3T3-E1 cells. *J. Biol. Chem.* 276: 7048–7054.
- Wadleigh, D. J., S. T. Reddy, E. Kopp, S. Ghosh, and H. R. Herschman. 2000. Transcriptional activation of the cyclooxygenase-2 gene in endotoxin-treated RAW 264.7 macrophages. *J. Biol. Chem.* 275: 6259–6266.
- Subbaramaiah, K., D. T. Lin, J. C. Hart, and A. J. Dannenberg. 2001. Peroxisome proliferator-activated receptor γ ligands suppress the transcriptional activation of cyclooxygenase-2: evidence for involvement of activator protein-1 and CREB-binding protein/p300. *J. Biol. Chem.* 276: 12440–12448.
- Subbaramaiah, K., L. Norton, W. Gerald, and A. J. Dannenberg. 2002. Cyclooxygenase-2 is overexpressed in HER-2/neu-positive breast cancer: evidence for involvement of AP-1 and PEA3. *J. Biol. Chem.* 277: 18649–18657.
- Chen, C. C., Y. T. Sun, J. J. Chen, and Y. J. Chang. 2001. Tumor necrosis factor- α -induced cyclooxygenase-2 expression via sequential activation of ceramide-dependent mitogen-activated protein kinases, and I κ B kinase 1/2 in human alveolar epithelial cells. *Mol. Pharmacol.* 59: 493–500.
- Huang, S. P., M. S. Wu, C. T. Shun, H. P. Wang, M. T. Lin, M. L. Kuo, and J. T. Lin. 2004. Interleukin-6 increases vascular endothelial growth factor and angiogenesis in gastric carcinoma. *J. Biomed. Sci.* 11: 517–527.
- Appleby, S. B., A. Ristimaki, K. Neilson, K. Narko, and T. Hla. 1994. Structure of the human cyclo-oxygenase-2 gene. *Biochem. J.* 302(Pt. 3): 723–727.
- Huang, W. C., and C. C. Chen. 2005. Akt phosphorylation of p300 at Ser-1834 is essential for its histone acetyltransferase and transcriptional activity. *Mol. Cell Biol.* 25: 6592–6602.
- Muta, T., and K. Takeshige. 2001. Essential roles of CD14 and lipopolysaccharide-binding protein for activation of Toll-like receptor (TLR)2 as well as TLR4: reconstitution of TLR2- and TLR4-activation by distinguishable ligands in LPS preparations. *Eur. J. Biochem.* 268: 4580–4589.
- Chen, C. C., M. P. Chow, W. C. Huang, Y. C. Lin, and Y. J. Chang. 2004. Flavonoids inhibit tumor necrosis factor- α -induced up-regulation of intercellular adhesion molecule-1 (ICAM-1) in respiratory epithelial cells through activator protein-1 and nuclear factor- κ B: structure-activity relationships. *Mol. Pharmacol.* 66: 683–693.
- Dannenberg, A. J., N. K. Altorki, J. O. Boyle, C. Dang, L. R. Howe, B. B. Weksler, and K. Subbaramaiah. 2001. Cyclo-oxygenase 2: a pharmacological target for the prevention of cancer. *Lancet Oncol.* 2: 544–551.
- Hao, C. M., M. Komhoff, Y. Guan, R. Redha, and M. D. Breyer. 1999. Selective targeting of cyclooxygenase-2 reveals its role in renal medullary interstitial cell survival. *Am. J. Physiol.* 277: F352–F359.
- Sheng, H., J. Shao, J. D. Morrow, R. D. Beauchamp, and R. N. DuBois. 1998. Modulation of apoptosis and Bcl-2 expression by prostaglandin E₂ in human colon cancer cells. *Cancer Res.* 58: 362–366.
- Huang, W. C., S. T. Chan, T. L. Yang, C. C. Tzeng, and C. C. Chen. 2004. Inhibition of ICAM-1 gene expression, monocyte adhesion and cancer cell invasion by targeting IKK complex: molecular and functional study of novel α -methylene- γ -butyrolactone derivatives. *Carcinogenesis* 25: 1925–1934.
- Tsujii, M., and R. N. DuBois. 1995. Alterations in cellular adhesion and apoptosis in epithelial cells overexpressing prostaglandin endoperoxide synthase 2. *Cell* 83: 493–501.
- Huang, M., M. Stolina, S. Sharma, J. T. Mao, L. Zhu, P. W. Miller, J. Wollman, H. Herschman, and S. M. Dubinett. 1998. Non-small cell lung cancer cyclooxygenase-2-dependent regulation of cytokine balance in lymphocytes and macrophages: up-regulation of interleukin 10 and down-regulation of interleukin 12 production. *Cancer Res.* 58: 1208–1216.
- Masferrer, J. L., K. M. Leahy, A. T. Koki, B. S. Zweifel, S. L. Settle, B. M. Woerner, D. A. Edwards, A. G. Flickinger, R. J. Moore, and K. Seibert. 2000. Antiangiogenic and antitumor activities of cyclooxygenase-2 inhibitors. *Cancer Res.* 60: 1306–1311.
- Mandell, L., A. P. Moran, A. Cocchiarella, J. Houghton, N. Taylor, J. G. Fox, T. C. Wang, and E. A. Kurt-Jones. 2004. Intact Gram-negative *Helicobacter pylori*, *Helicobacter felis*, and *Helicobacter hepaticus* bacteria activate innate immunity via Toll-like receptor 2 but not Toll-like receptor 4. *Infect. Immun.* 72: 6446–6454.
- Smith, M. F., Jr., A. Mitchell, G. Li, S. Ding, A. M. Fitzmaurice, K. Ryan, S. Crowe, and J. B. Goldberg. 2003. Toll-like receptor (TLR) 2 and TLR5, but not TLR4, are required for *Helicobacter pylori*-induced NF- κ B activation and chemokine expression by epithelial cells. *J. Biol. Chem.* 278: 32552–32560.
- Ding, S. Z., A. M. Torok, M. F. Smith, Jr., and J. B. Goldberg. 2005. Toll-like receptor 2-mediated gene expression in epithelial cells during *Helicobacter pylori* infection. *Helicobacter* 10: 193–204.
- Schmausser, B., M. Andrusis, S. Endrich, S. K. Lee, C. Josenhans, H. K. Muller-Hermelink, and M. Eck. 2004. Expression and subcellular distribution of Toll-like receptors TLR4, TLR5 and TLR9 on the gastric epithelium in *Helicobacter pylori* infection. *Clin. Exp. Immunol.* 136: 521–526.
- Pinhal-Enfield, G., M. Ramanathan, G. Hasko, S. N. Vogel, A. L. Salzman, G. J. Boons, and S. J. Leibovich. 2003. An angiogenic switch in macrophages involving synergy between Toll-like receptors 2, 4, 7, and 9 and adenosine A^{2A} receptors. *Am. J. Pathol.* 163: 711–721.
- DuBois, R. N., F. M. Giardiello, and W. E. Smalley. 1996. Nonsteroidal anti-inflammatory drugs, eicosanoids, and colorectal cancer prevention. *Gastroenterol. Clin. North Am.* 25: 773–791.
- Wang, W. H., J. Q. Huang, G. F. Zheng, S. K. Lam, J. Karlberg, and B. C. Wong. 2003. Non-steroidal anti-inflammatory drug use and the risk of gastric cancer: a systematic review and meta-analysis. *J. Natl. Cancer Inst.* 95: 1784–1791.
- Langman, M. J., K. K. Cheng, E. A. Gilman, and R. J. Lancashire. 2000. Effect of anti-inflammatory drugs on overall risk of common cancer: case-control study in general practice research database. *Br. Med. J.* 320: 1642–1646.
- Juttner, S., T. Cramer, S. Wessler, A. Walduck, F. Gao, F. Schmitz, C. Wunder, M. Weber, S. M. Fischer, W. E. Schmidt, et al. 2003. *Helicobacter pylori* stimulates host cyclooxygenase-2 gene transcription: critical importance of MEK/

- ERK-dependent activation of USF1/-2 and CREB transcription factors. *Cell Microbiol.* 5: 821–834.
45. Karin, M., Z. Liu, and E. Zandi. 1997. AP-1 function and regulation. *Curr. Opin. Cell Biol.* 9: 240–246.
 46. Han, S., H. Inoue, L. C. Flowers, and N. Sidell. 2003. Control of COX-2 gene expression through peroxisome proliferator-activated receptor γ in human cervical cancer cells. *Clin. Cancer Res.* 9: 4627–4635.
 47. Tang, Q., W. Chen, M. S. Gonzales, J. Finch, H. Inoue, and G. T. Bowden. 2001. Role of cyclic AMP responsive element in the UVB induction of cyclooxygenase-2 transcription in human keratinocytes. *Oncogene* 20: 5164–5172.
 48. Chang, Y. J., C. Y. Liu, B. L. Chiang, Y. C. Chao, and C. C. Chen. 2004. Induction of IL-8 release in lung cells via activator protein-1 by recombinant baculovirus displaying severe acute respiratory syndrome-coronavirus spike proteins: identification of two functional regions. *J. Immunol.* 173: 7602–7614.
 49. Meyer-ter-Vehn, T., A. Covacci, M. Kist, and H. L. Pahl. 2000. *Helicobacter pylori* activates mitogen-activated protein kinase cascades and induces expression of the proto-oncogenes *c-fos* and *c-jun*. *J. Biol. Chem.* 275: 16064–16072.
 50. Mayr, B., and M. Montminy. 2001. Transcriptional regulation by the phosphorylation-dependent factor CREB. *Nat. Rev. Mol. Cell Biol.* 2: 599–609.
 51. Yu, C. T., H. M. Shih, and M. Z. Lai. 2001. Multiple signals required for cyclic AMP-responsive element binding protein (CREB) binding protein interaction induced by CD3/CD28 costimulation. *J. Immunol.* 166: 284–292.
 52. Tsai, E. Y., J. Yie, D. Thanos, and A. E. Goldfeld. 1996. Cell-type-specific regulation of the human tumor necrosis factor α gene in B cells and T cells by NFATp and ATF-2/JUN. *Mol. Cell Biol.* 16: 5232–5244.
 53. van Dan, H., M. Duyndam, R. Rottier, A. Bosch, L. de Vries-Smits, P. Herrlich, A. Zantema, P. Angel, and A. J. van der Eb. 1993. Heterodimer formation of cJun and ATF-2 is responsible for induction of *c-jun* by the 243 amino acid adenovirus E1A protein. *EMBO J.* 12: 479–487.