Original Article

Helicobacter pylori Induces Promoter Methylation of E-cadherin via Interleukin-1β Activation of Nitric Oxide Production in Gastric Cancer Cells

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BACKGROUND: *Helicobacter pylori* infection causes gastric mucosal inflammatory responses, resulting in up-regulation of interleukin-1β (IL-1β) and overproduction of mutagenic nitric oxide (NO). The authors previously demonstrated that IL-1β plays an important role in *H. pylori*-induced E-cadherin (E-cad) methylation. Here, they extend the study to investigate the downstream effect of IL-1β on *H. pylori*-induced gastric inflammation and aberrant DNA methylation. **METHODS:** Human gastric cancer cell lines (MKN7, MKN74, and TMK-1) with and without pretreatment of IL-1 receptor antagonist (IL-1ra) were treated with IL-1β or infected with *H. pylori*. Promoter methylation status of E-cad was examined by methylation-specific polymerase chain reaction (PCR). Expression of E-cad, inducible nitric oxide synthase (iNOS), and nuclear factor κB (NFκB) was assessed by quantitative reverse transcriptase PCR, Western blotting, or immunofluorescence. NO production and total DNA methyltransferase (DNMT) activity were assayed fluorometrically. **RESULTS:** Both IL-1β treatment and *H. pylori* infection-induced E-cad methylation led to a decrease in E-cad expression at both mRNA and protein levels. Total DNMT enzymatic activity was significantly elevated in treated cells, accounting for the observed E-cad methylation induction. Increased expression of NFκB was accompanied by up-regulation of iNOS and production of NO in treated cells. Reversal of all these phenomena in cells pretreated with IL-1ra suggested *H. pylori*-induced E-cad methylation via IL-1β stimulation of the NFκB transcriptional system, leading to activation of DNMT activity by NO production. **CONCLUSIONS:** These findings reveal a previously unknown effect of IL-1β and NO on *H. pylori*-induced aberrant DNA methylation. This possible pathway indicates the role of NO in epigenetic modification that links inflammation to carcinogenesis. ***Cancer*** **2012;000:000–000.** © *2012 American Cancer Society.*

KEYWORDS: gastric cancer, *Helicobacter pylori*, methylation, E-cadherin, carcinogenesis.

INTRODUCTION

Helicobacter pylori is recognized as one of the most common human infections, and about half of the world's population carries this organism.[1] Chronic mucosal inflammation caused by persistent *H. pylori* infection is linked to an increased risk of malignant transformation in gastric epithelium cells.[2,3] However, the mechanisms whereby *H. pylori* escapes the host defense system and causes gastric diseases are still not clear.

Host immune response to *H. pylori* infection includes local infiltration of neutrophils and macrophages and activation of nuclear factor κB (NFκB) in the gastric mucosa cells.[4] NFκB plays a critical role in the inflammatory process, as it regulates transcription of a wide variety of inflammatory genes, such as cytokines, chemokines, and adhesion molecules.[5] One of the key cytokines up-regulated in *H. pylori* infection is interleukin-1β (IL-1β), which is important in initiating and amplifying the inflammatory responses against the invading bacteria.[6-8] In addition, *H. pylori* infection also stimulates expression of inducible nitric oxide synthase (iNOS) in the invading macrophages as well as in the gastric mucosa.[9] Generation of nitric oxide (NO) by iNOS activation is a common strategy by the host to eradicate *H. pylori* upon stimulation by its toxins.[10] However, the survival of *H. pylori* and its persistent colonization in the gastric mucosa suggest that the bacteria have developed mechanisms to avoid NO-dependent killing and maintain an active chronic inflammatory reaction that causes chronic gastritis and peptic ulcer disease.[11,12] It is still not known how this local inflammatory response leads to cancer, but overproduction of the mutagenic NO can induce irreversible mucosal DNA damage that has been proposed to be involved in the initiation and promotion of tumor growth.[13]

Silencing of tumor suppressor genes by promoter hypermethylation plays a crucial role in tumorigenesis. Understanding its underlying mechanism is essential for cancer prevention and treatment. Mounting evidence suggests that *H. pylori* infection induces aberrant promoter methylation in many tumor suppressor genes during the multistep process

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of gastric carcinogenesis.[14-16] In particular, methylation-dependent silencing of tumor suppressor gene E-cadherin (E-cad) is reported to be an early event in human gastric carcinogenesis.[17,18] We have previously studied the role of IL-1β in *H. pylori*-induced E-cad methylation and demonstrated that *H. pylori*-caused E-cad methylation is mediated through activation of IL-1β production.[19,20] We also demonstrated that methylation of E-cad gene occurred in *H. pylori*-infected non-neoplastic gastric mucosa, and methylation can be reversed after successful *H. pylori* eradication.[21] However, the underlying mechanism of DNA methylation and the reversal of the methylation process are still unclear.

Chronic inflammation is known to promote certain types of cancers, such as liver and gastric cancers. Chronic inflammation and aberrant DNA methylation are frequently observed in *H. pylor*i-associated gastric diseases. Hence, chronic inflammation caused by *H. pylor*i infection is suggested to be an inducer of aberrant DNA methylation. Because of the reversibility of methylation and eradication of *H. pylori* infection, it is extremely important to explore the link between DNA methylation and inflammation. In this study, we used human gastric cancer cell lines to elucidate the underlying mechanism of gastric inflammation triggered by *H. pylori* infection in induction of aberrant DNA methylation.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

The 2 human gastric cancer cell lines MKN7 and MKN74 were purchased from the Cell Resource Bank (Ibaraki, Japan). TMK-1 cells were obtained from Dr. Eiichi Tahara (University of Hiroshima, Hiroshima, Japan).[22] Cells were cultured in Dulbecco modified Eagle medium containing 10% heat-inactivated fetal bovine serum supplemented with penicillin (100 U/mL) and streptomycin (100 μg/mL). Cells were grown at 37°C under a humidified atmosphere with 5% CO_2 in air.

Culture With IL-1β and Blockage by Receptor Antagonist

For treatment, a total of 1×10^6 gastric cancer cells per well were cultured in medium containing 2.5 ng/mL of recombinant human IL-β (rhIL-1β) (R&D Systems, Minneapolis, Minn) for 6 hours for TMK-1 and MKN74, and 24 hours for MKN7, as previously described.[20] The cells were pretreated with or without 20 ng/mL of human IL-1 receptor antagonist (IL-1ra) for 1 hour before cell harvesting for analysis (R&D Systems). All culture supernatants were collected for NO measurement.

H. pylori Culture and Gastric Cell Infection

H. pylori Sydney strain 1 (*H. pylori*-SS1) were used. This strain processes the virulent cagA+ and vacA s2-m2 proteins. The bacteria were cultured on 7% horse blood agar plates (Columbia agar base; Oxoid, Basingstoke, UK) in microaerophilic conditions, and harvested after 2 to 3 days. The bacteria were quantified by absorbance at 660 nm ($1\ OD_{660nm} = 1 \times 10^8$ colony-forming units/mL). Then, the gastric cells were cocultured with *H. pylori* at a ratio of 10:1 to TMK-1 for 2 days or MKN74 for 3 days; and at a ratio of 100:1 to MKN7 for 4 days, as described in our previous study.[20] The cells were grown at 37°C in a humidified atmosphere before being collected. Again, cells were pretreated with or without 20 ng/mL of IL-1ra for 1 hour (R&D Systems). All culture supernatants were collected for further NO analysis.

Genomic DNA Isolation and Bisulfite Modification

Genomic DNA was isolated from cells by High-Pure PCR Template Preparation Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. Bisulfite modification of DNA samples was performed using the CpGenome DNA modification kit (Intergen, Purchase, NY). Briefly, 1 μg of genomic DNA from each sample was denatured with 3 mol/L NaOH at 37°C for 10 minutes, followed by incubation with sodium bisulfate (pH 5.0) at 50°C for 16 hours. After modification, the DNA was eluted into 40 μL Tris-ethylenediamine tetraacetic acid. The modified DNA was used immediately or stored at −20°C for further analysis.

Methylation-Specific Polymerase Chain Reaction

Aliquots of bisulfite-modified DNA (1 μL) were used as templates for methylation-specific polymerase chain reaction (PCR). The primer sequences specific for promoter methylated or unmethylated E-cad gene and PCR conditions were described in our previous study.[17] CpGenome Universal Methylated DNA (Intergen) and reagent blanks were used as positive and negative controls, respectively, in each experiment. PCR products were electrophoresed in 2% agarose gel, stained with ethidium bromide, and observed under a ultraviolet illuminator. All tests were performed in duplicate. For confirmation of the specificity of the methylation-specific PCR, PCR products were purified and sequenced as previously described.[17]

Measurement of NO Production

The transient and volatile nature of NO makes it unsuitable for a convenient detection method. Nitrate, a stable end product of NO, was determined in culture supernatants by a spectrophotometric method based on the Griess

Table 1. Primer Sequences Used for Semiquantitative RT-PCR (RT-PCR) and Quantitative Real Time RT-PCR (Q-PCR) Analysis

Gene	Forward Primer (5′ → 3′)	Reverse Primer (5′ → 3′)
E-cad	GGAATCCAAGCAGAAGC	TATGTGGCAATGCGTTCTTATCCA
iNOS[a]	ACAAGCTGGCCTCGCTCTGGAAAGA	TCCATGCAGACAACCTTG GGGTTGAAG
iNOS	CCCGAGTCAGAGTCACCATCC	TCAAACGTCTCACAGGCTGCC
GAPDH	CAAATTCCATGGCACCGTCA	TCTCGCTCCTGGAAGATG GTGA

Abbreviations: E-cad, E-cadherin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; iNOS, inducible nitric oxide synthase; PCR, polymerase chain reaction.
[a] Primer sequences used in semiquantitative reverse transcriptase PCR analysis.

reaction using the total NO assay kit (Enzo Life Science, Plymouth Meeting, Pa). Measurements were performed according to the manufacturer's instructions. Briefly, 50 μL of sample supernatant was plated in 96-well flat-bottomed plates and incubated first with 25 μL each of nicotinamide adenine dinucleotide and nitrate reductase dilution at 37°C for 30 minutes. This was followed by incubation with 50 μL each of Griess reagents I and II at room temperature for 10 minutes. The optical density was measured at 550 nm using a microplate reader (Molecular Devices, Sunnyvale, Calif). All standards and samples were measured in triplicate experiments.

Semiquantitative and Quantitative Reverse Transcriptase PCR Analysis

Total RNAs were isolated from the cell lines with TRIzol reagent (Invitrogen, Carlsbad, Calif). mRNA was quantified and first-strand cDNA was synthesized using the Superscript II-reverse transcriptase kit (Invitrogen). Semiquantitative reverse transcriptase PCR (RT-PCR) was performed as previously described using AmpliTaq-Gold (Applied Biosystems, Foster City, Calif), with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control to ensure the quality and quantity for each RT-PCR. Quantitative real time RT-PCR (Q-PCR) was performed in a final volume of 20 μL containing 1 μL of cDNA samples, 200 μM of each of the primers, and 5 μL of SYBR green PCR master mix (Roche). The relative fold expression changes were calculated using the $2^{-\Delta\Delta CT}$ method. Data were obtained from 3 individual experiments and normalized with the control *GAPDH* gene. Primer sequences for the amplification are listed in Table 1.

Western Blotting Analysis

After treatments, cells were washed twice with 1 × ice-cold phosphate-buffered saline (PBS) and lysed in RIPA buffer (Roche). The protein concentrations were determined using the Bradford method (Bio-Rad Laboratories, Hercules, Calif). Balanced amounts of the cell proteins were loaded onto a 7.5% to 10% sodium dodecyl sulfate-polyacrylamide gel to be separated by electrophoresis followed by transfer onto a nitrocellulose membrane (GE Healthcare, Piscataway, NJ). The membrane was blocked with 5% milk solution and incubated with primary antibodies of E-cad (1:500; Calbiochem, San Diego, Calif), NFκB-p50/p65 (1:500), and iNOS (1:250; Santa Cruz Biotechnology, Santa Cruz, Calif) overnight at 4°C, respectively. Anti–β-actin antibody (1:1000) was used as a loading control. The membranes were washed in Tris-buffered saline and 0.05% Tween 20 and incubated with secondary antibody for 1 hour at room temperature. The signals were developed with the ECL kit (GE Healthcare). The band intensity was quantified by ImageJ software (National Institutes of Health, Bethesda, Md).

Double Immunofluorescence Analysis

Cells were fixed with 4% paraformaldehyde (Sigma, St Louis, Mo). Nonspecific binding was blocked by incubating cells in the blocking buffer (10% normal donkey serum and 0.3% triton X-100 in PBS solution) for 45 minutes at room temperature. Cells were incubated overnight at 4°C with first primary antibody solution and then washed 3× with PBS and incubated for 1 hour at room temperature with the corresponding first secondary antibody. Then cells were washed and incubated overnight at 4°C with second primary antibody solution and then washed and incubated for 1 hour at room temperature with the corresponding second secondary antibody. Antibodies used were as follows: rat monoclonal anti–E-cad (1:1000; Abcam, Cambridge, UK); rabbit polyclonal anti–NFκB-p50/65 (1:500; Abcam) in 0.1% bovine serum albumin solution; and Alexa Fluor 568 donkey antirabbit, and Alexa Fluor 488 donkey antirat (all 1:200 in PBS, from Molecular Probes, Eugene, Ore and Invitrogen). For nuclear labeling, cell preparations were stained with antifade reagent with DAPI. Fluorescent images were acquired using a fluorescent microscope (AxioVision, Zeiss, Germany).

Measurement of DNA Methyltransferase Activity

Cells with or without treatment as described above were harvested, and nuclear protein extract was obtained according to the manufacturer's instructions using the

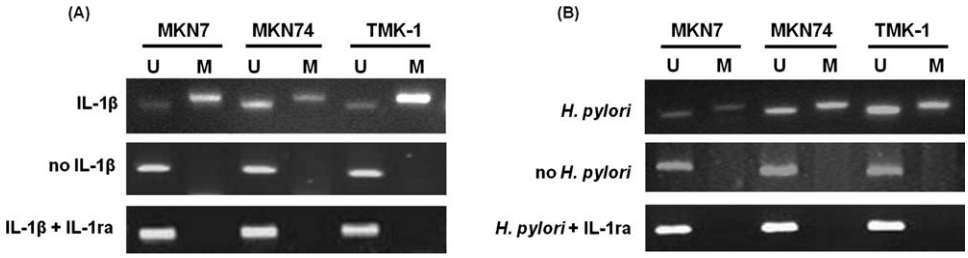

Figure 1. *H. pylori* infection or interleukin-1β (IL-1β) challenge induced promoter methylation of E-cadherin (E-cad) in gastric cancer cells. MKN7, MKN74, and TMK-1 cells were treated with IL-1β (A) or cocultured with *H. pylori* (B) as described in the Materials and Methods section. Pretreatment of cells with or without IL-1 receptor antagonist (IL-1ra) was also performed. Promoter methylation status of E-cad was detected by methylation-specific polymerase chain reaction. U, unmethylated DNA; M, methylated DNA.

EpiQuik nuclear extraction kit (Epigentek, Brooklyn, NY). Protein concentrations were determined using the Bradford method (Bio-Rad Laboratories). The total DNA methyltransferase (DNMT) activity was measured using the EpiQuik DNA methyltransferase activity assay kit (Epigentek). In this assay, the cytosine-rich DNA substrate is stably coated on the strip wells. These wells are specifically treated to confer a high DNA adsorption ability. DNMT transfers a methyl group from S-adenosylmethionine to a cytosine in the DNA substrate. Methylated DNA can be recognized using an anti–5-methylcytosine antibody. The levels of methylated DNA, which were proportional to the enzymatic activity, were then colorimetrically quantified using an enzyme-linked immunosorbent assaylike reaction. Absorbance was determined using a microplate spectrophotometer at 450 nm, and DNMT activity (OD/h/mg) was calculated according to the manufacturer's instructions. Results are given in activity units expressed relative to the activity level detected in control cells.

Statistical Analysis

Comparisons among different groups were performed by Student *t* test using (GraphPad Software, La Jolla, Calif). Statistical significance was set at $P < .05$.

RESULTS

IL-1β or H. pylori Challenge Induced E-cad Methylation

To study the effect of IL-1β on promoter methylation of E-cad, the 3 gastric cancer cell lines (MKN7, MKN74, and TMK-1) were treated with IL-1β, and methylation status of E-cad was analyzed by methylation-specific PCR. Methylation of E-cad was observed in all cells treated with IL-1β (Fig. 1A). No methylation of E-cad gene was observed in control cells without IL-1β treatment. To confirm that the methylation of E-cad was specifically because of IL-1β stimulation, cells were pretreated with IL-1ra. No methylated alleles were present in the 3 cancer cell lines after pretreatment with IL-1ra (Fig. 1A). Promoter methylation of E-cad was also induced in cells upon cocultured with *H. pylori*-SS1 (Fig. 1B). No methylation alleles were observed in the control cells without *H. pylori* challenge. Similarly, methylation disappeared when the cells were pretreated with the antagonist IL-1ra. These results suggest that both IL-1β and *H. pylori* induced promoter methylation of E-cad, and *H. pylori* possibly acts through an IL-1β-dependent pathway. Sequencing analysis on methylation-specific PCR products confirmed CpG sites within the E-cad promoter region were methylated (data not shown).

H. pylori Infection or IL-1β Challenge Induced NO Production

Overproduction of NO causes DNA damage in *H. pylori*-associated gastric diseases. We then assessed the effect of IL-1β stimulation and *H. pylori* infection on NO production (evaluated as nitrate). As shown in Figure 2, cells at a density of 1×10^6/well challenged with IL-1β produced significantly higher concentration of NO (43.0 ± 3.2 μM in MKN7 cells, 13.9-fold increase above control level of 3.1 ± 2.2 μM, $P < .0001$; 51.3 ± 1.9 μM in MKN74, 5.5-fold increase above control level of 9.3 ± 2.2 μM, $P < .005$; 55.3 ± 2.3 μM in TMK-1, 5.2-fold increase above control level of 10.7 ± 1.5 μM, $P < .005$) than the respective control cells. Similarly, *H. pylori* infection also stimulated an increase in NO production in treated cells (28.7 ± 1.9 μM in MKN7 cells, 9.3-fold increase above control level of 3.1 ± 2.2 μM, $P < .0001$; 31.7 ± 2.3 μM in MKN74, 3.4-fold increase above control level of 9.3 ± 2.2 μM, $P < .005$; 39.0 ± 1.2 μM in TMK-1, 3.6-fold increase above control level of 10.7 ± 1.5 μM, $P < .005$). To explore the role of IL-1β in *H. pylori* induction of NO production, cells were pretreated with IL-1ra. The results showed that IL-1ra significantly abolished the induction effect of IL-1β and *H. pylori* on NO production, and NO

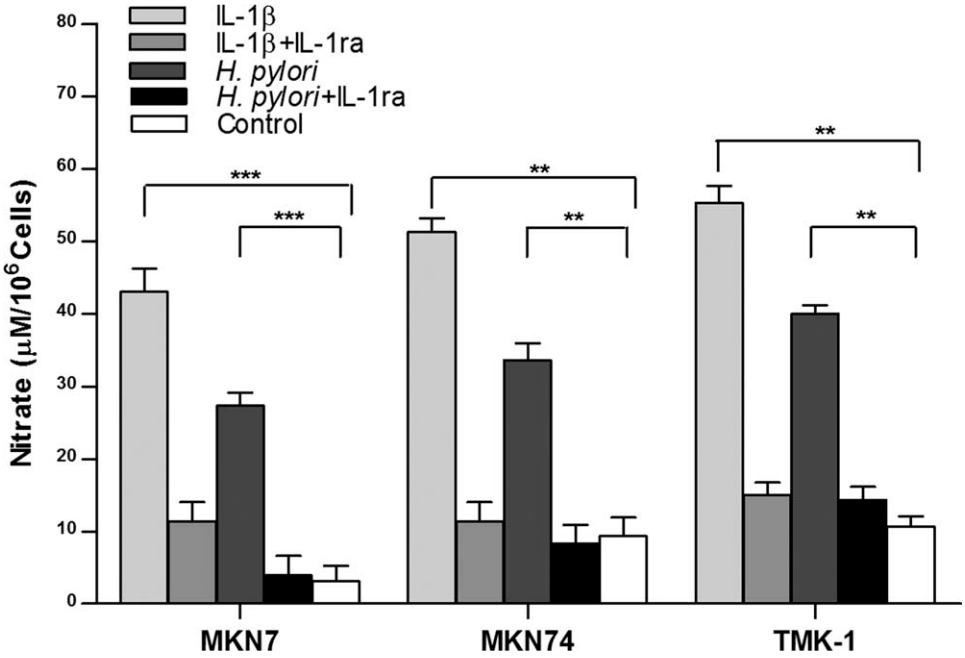

Figure 2. Measurement of nitric oxide (NO) production in gastric cancer cells was assessed by nitrate in culture supernatant based on the Griess method. NO levels were significantly elevated in cells challenged with interleukin-1β (IL-1β) or cocultured with *H. pylori* when compared with untreated control cells. Pretreatment of cells with IL-1 receptor antagonist (IL-1ra) abolished the induction effect of *H. pylori* and IL-1β on NO production. Columns and bars show the mean ± standard error of the mean. Statistically significant differences compared with control culture without any treatment are denoted by asterisks above the bars: ***$P <$ .0001, **$P <$ .005.

concentration measured was relatively similar to the control cells (Fig. 2). These data suggest that both IL-1β and *H. pylori* were able to induce NO production in gastric cancer cell lines, and *H. pylori* possibly induced NO production through the action of IL-1β.

Alteration of E-cad and iNOS mRNA Expression

We then determined the mRNA expression levels of E-cad and iNOS in cells treated with IL-1β or cocultured with *H. pylori* using semiquantitative RT-PCR, and the band intensity (arbitrary units) for each gene was quantified. As shown in Figure 3, upon IL-1β treatment, the expression of E-cad was significantly down-regulated in MKN7 and TMK-1 cells (1.6-fold and 1.5-fold reduction compared with unchallenged controls, respectively; both $P <$ .005), but not in MKN74 cells ($P =$ .078). For cells with *H. pylori* infection, mRNA expression of E-cad was also down-regulated (1.8-fold in MKN7, 1.3-fold in MKN74, and 2-fold in TMK-1; all $P <$ .005; Fig. 3A and C). In contrast, stimulation of the cells with IL-1β or *H. pylori* resulted in significantly increased in iNOS mRNA expression, almost 2-fold increase in expression was found in MKN7cells (both $P <$ .005), approximately 2.5-fold increase was found in MKN74 cells (both $P <$ .0001), and at least 1.5-fold increase was detected in TMK-1 cells (both $P <$ .005; Fig. 3B and D). RT-PCR was also performed after pretreatment of the cells with IL-1ra. The results showed that both E-cad and iNOS mRNA expression returned to levels comparable to the control cells.

Because of the more pronounced differences in gene expression after IL-1β and *H. pylori* treatment, and to confirm our hypothesis that *H. pylori* altered E-cad gene expression via activation of IL-1β, we further checked the expression of genes by Q-PCR. The use of the $2^{-\Delta\Delta CT}$ calculation revealed that the relative alteration in E-cad mRNA expression level in IL-1β-treated or *H. pylori*-treated cells in relation to untreated cells was similar to the results obtained using semiquantitative RT-PCR method. However, using Q-PCR, iNOS mRNA expression level in treated cells was basically higher than data obtained using the semiquantitative RT-PCR method (Fig. 3E). In detail, relative fold reduction of E-cad gene was detected upon IL-1β or *H. pylori* treatment (IL-1β treatment: 1.49 for MKN7, 1.12 for MKN74, and 1.57 for TMK-1; *H. pylori* infection: 2.2 for MKN7, 2.86 for MKN74, and 1.79 for TMK-1). Stimulation of the cells with IL-1β or *H. pylori* resulted in fold induction of iNOS mRNA expression (IL-1β treatment: 4.67 for MKN7, 4.08 for

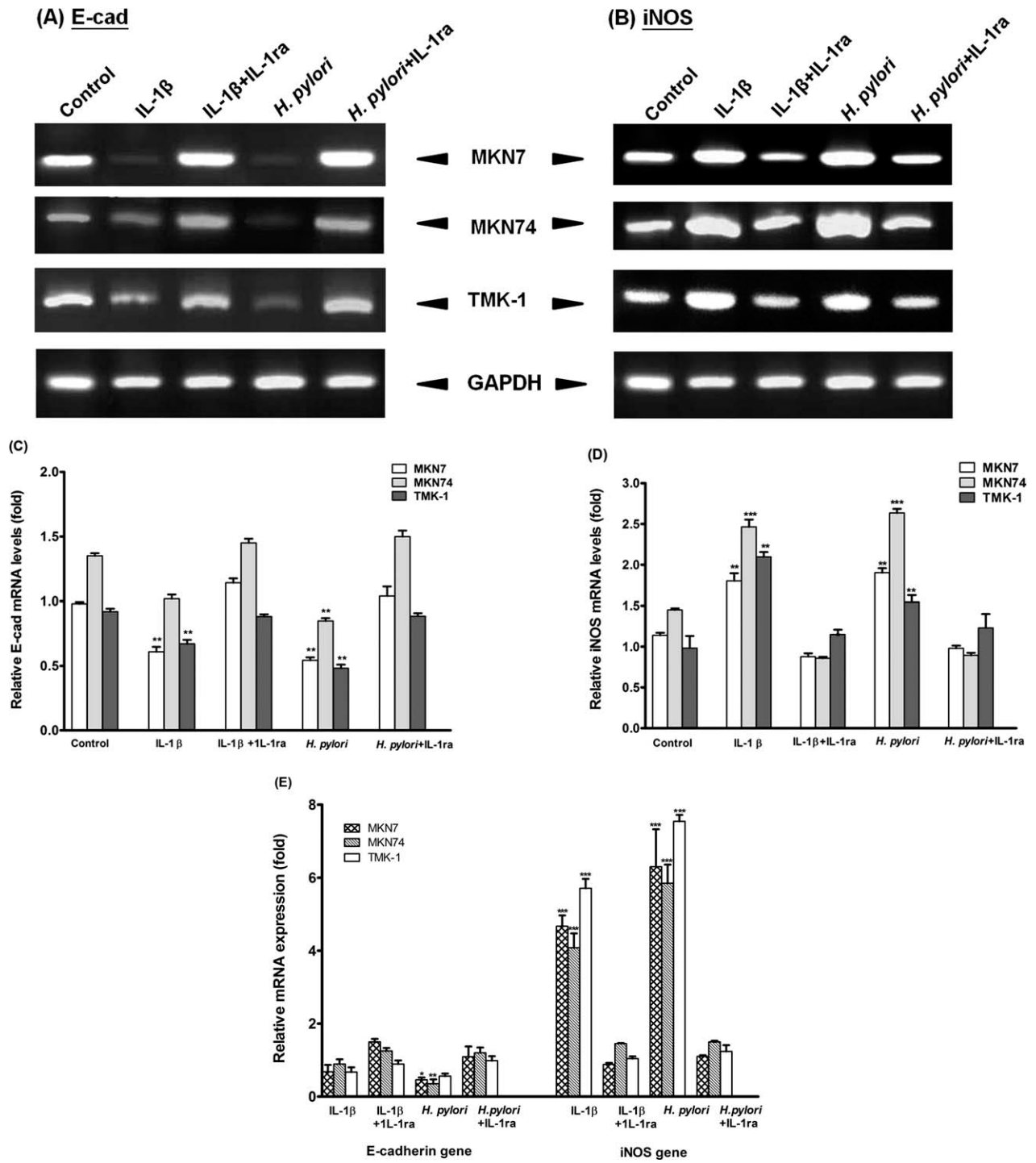

Figure 3. Semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR) analysis showed that mRNA expression of E-cadherin (E-cad; A and C) was suppressed and inducible nitric oxide synthase (iNOS; B and D) was up-regulated in interleukin-1β (IL-1β)-treated or *H. pylori*-treated gastric cancer cells. The expression levels of E-cad and iNOS returned to levels comparable to the controls when cells were pretreated with IL-1 receptor antagonist (IL-1ra). The arbitrary values were normalized to the control gene (GAPDH) and were expressed as mean ± standard error of the mean fold over respective control cells from 4 independent experiments. (E) Quantitative real time RT-PCR (Q-PCR) analysis of changes in E-cad and iNOS expression levels as described under in Materials and Methods. The relative fold differences were calculated using the $2^{-\Delta\Delta CT}$ method. Statistically significant differences are denoted by asterisks above the bars: ***$P < .0001$, ** $P < .005$, *$P < .01$).

MKN74, and 5.71 for TMK-1; *H. pylori* infection: 6.3 for MKN7, 5.85 for MKN74, and 7.54 for TMK-1). The relative reduction of E-cad expression and elevation of iNOS expression ascertained by Q-PCR agreed with the semiquantitative RT-PCR analysis.

Protein Expression of E-cad, iNOS, and NFκB

NFκB plays a critical role in the coordination of both IL-1β and iNOS expression during inflammatory response to infection. We next examined the protein expression levels of NFκB, iNOS, and E-cad in the 3 cells. As shown in Figure 4, in both IL-1β-treated and *H. pylori*-treated cells, there was a great increase in iNOS protein expression (2-fold to 5-fold), whereas the expression of E-cad protein was down-regulated (approximately 2-fold). These findings were similar in degree to the mRNA expression analysis of E-cad and iNOS shown in Figure 3. Again, when the cells were pretreated with IL-1ra, the effects were diminished, and protein expression returned to levels comparable to control cells (Fig. 4). There was no significant difference in expression of the two NFκB functional subunits in individual cell lines, although the expression level for both proteins was found to be relatively higher in MKN74 cells (Fig. 4). This may explain the significant up-regulation of iNOS mRNA levels shown in Figure 3D ($P < .0001$), and thus the high level of NO production shown in Figure 2 ($P < .005$). Specifically, the findings of the increase in expression of NFκB and iNOS, which parallels the increase in NO production, indicate that NO is produced from the iNOS activation.

Immunofluorescence Analysis of Gene Expression

To further assess the role of NFκB in activation of inflammatory gene expression and to determine whether the differences observed in E-cad mRNA expression levels translated into significant alterations of protein expression pattern, we performed immunofluorescence analysis for E-cad and NFκB-p50/p65 expression. As expected, IL-1β treatment or *H. pylori* infection induced the translocation of NFκB-p65 subunit from the cytoplasm into the nucleus (Fig. 5). We observed no significant difference in fluorescence intensity between cytosolic and nucleic NFκB-p50 subunit upon both treatments (data not shown). These observations were in line with the Western blot results illustrating expression changes in cytosolic and nucleic NFκB protein subunits. Although down-regulation of mRNA and protein expression of E-cad was detected, no significant characteristic change in the fluorescence intensity of E-cad was detected upon treatments. However, infection with *H. pylori* tends to destabilize epithelial cell adherence, causing internalization of E-cad gene and the formation of intracellular vesicles (Fig. 5) in these 3 gastric cancer cells.

Activation of Total DNMT Enzymatic Activity

DNMTs are responsible for maintenance and induction of DNA methylation, and their activation is frequently observed in various types of human cancer.[23] We measured the total DNMT enzymatic activity to further define the effects of *H. pylori* infection and IL-1β on induction of E-cad methylation. As shown in Figure 6, there was a significant increase in the DNMT activity in cells challenged with IL-1β or *H. pylori*. Approximately 3-fold and 4-fold increase in DNMT activity was found in MKN7 and MKN74 with both challenges ($P < .01$; $P < .05$), respectively. Up to a 6-fold increase was found in TMK-1 cells treated with IL-1β, and a 4.5-fold increase was detected in *H. pylori*-cocultured TMK-1 cells (both $P < .0001$). Pretreatment of the cells with blocker IL-1ra returned the DNMT activity to levels comparable to control cells (Fig. 6). These observations indicate that both *H. pylori* and IL-1β could induce DNMT enzymatic activity, and *H. pylori* modulates host gene methylation probably through an IL-1β-dependent pathway.

DISCUSSION

Although studies have demonstrated that *H. pylori* infection inducing tumor suppressor gene silencing by DNA hypermethylation plays a crucial role in cancer development and progression, the underlying mechanism of methylation induction remains largely unexplored. In this study, we have identified overproduction of NO as one of the possible pathways of *H. pylori*-induced aberrant DNA methylation. We first demonstrated that both IL-1β and *H. pylori* challenges induced promoter methylation of E-cad gene in gastric cancer cells. Semiquantitative RT-PCR method was then adopted to detect the effects of DNA methylation on gene expression. The result indicated that mRNA expression of E-cad was significantly down-regulated, whereas iNOS expression was up-regulated in comparison with their corresponding untreated controls. The changes in E-cad and iNOS expression level were also confirmed quantitatively by real-time PCR. The results confirmed that methylation of E-cad gene led to reduction in mRNA expression, and IL-1β and *H. pylori* challenges induced iNOS mRNA expression. We suggest that *H. pylori* induced E-cad methylation via the activation of iNOS expression.

The activation of the transcription factor NFκB has been identified as an essential requirement for the

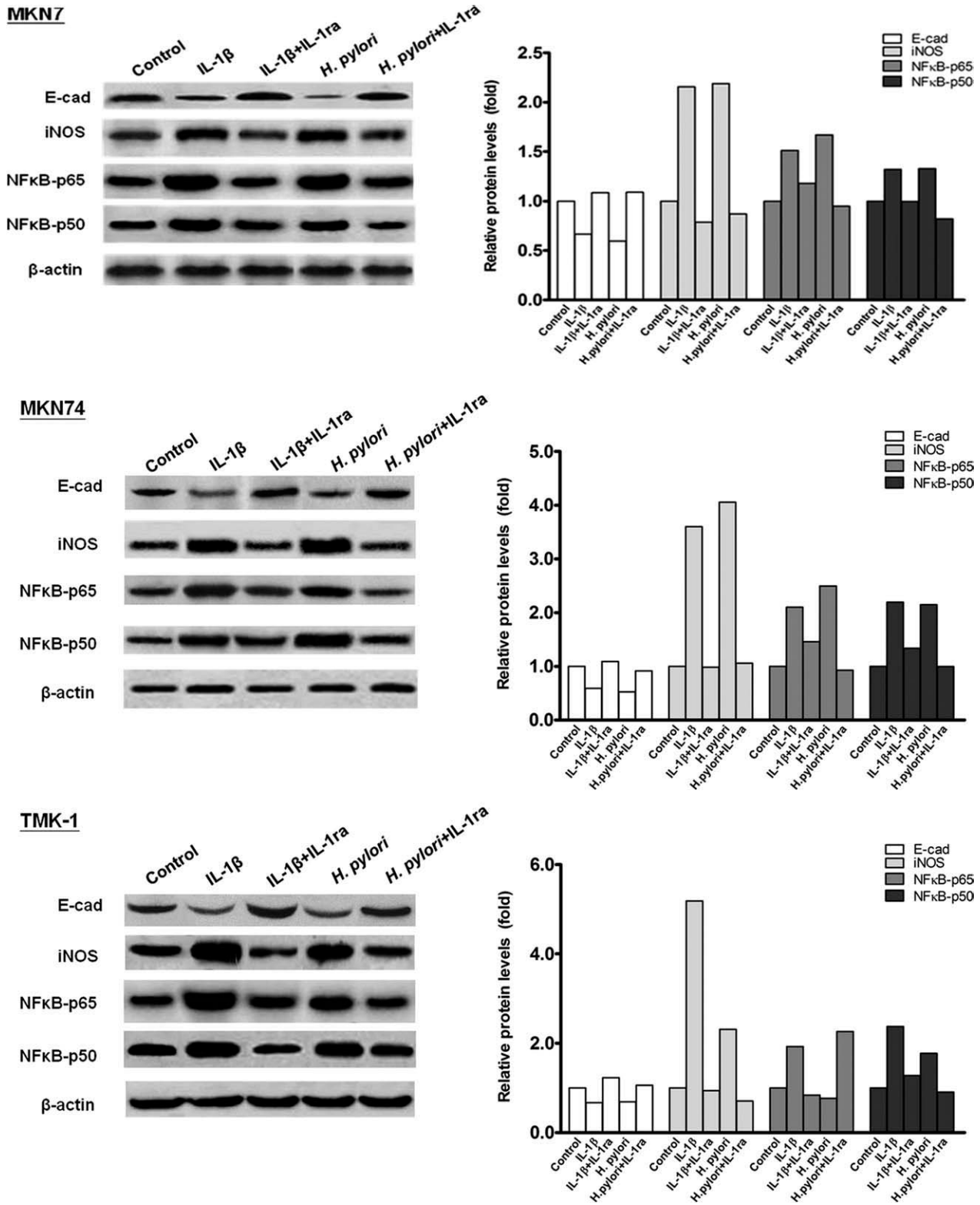

Figure 4. Both interleukin-1β (IL-1β) and *H. pylori* challenge suppressed E-cadherin (E-cad) protein expression but stimulated nuclear factor κB (NFκB) and inducible nitric oxide synthase (iNOS) expression in gastric cancer cells. Representative Western blots of MKN7, MKN74, and TMK-1 cells treated with IL-1β or cocultured with *H. pylori* or cells pretreated with IL-1 receptor antagonist (IL-1ra) are shown. The arbitrary values were obtained relative to respective controls and human β-actin in each group of cancer cells.

expression of iNOS. Thus, we analyzed the protein expression pattern of E-cad, iNOS, and NFκB-p50/p65 subunits by Western blot. We found that expression of E-cad and iNOS proteins was in accord with their mRNA expression, and NFκB-p50/p65 subunits protein expression was up-regulated in cells challenged with IL-1β or cocultured with *H. pylori*. Interestingly, we found there was no difference in cytosolic and nucleic NFκB-p50 protein expression. Although nucleic NFκB-p65 protein expression was elevated in cells with IL-1β treatment or *H. pylori* challenge, the level of change was not significant (data not shown). We believe *H. pylori* infection activates IL-1β production, which then stimulates the NFκB transcriptional system, resulting in NO/iNOS activation

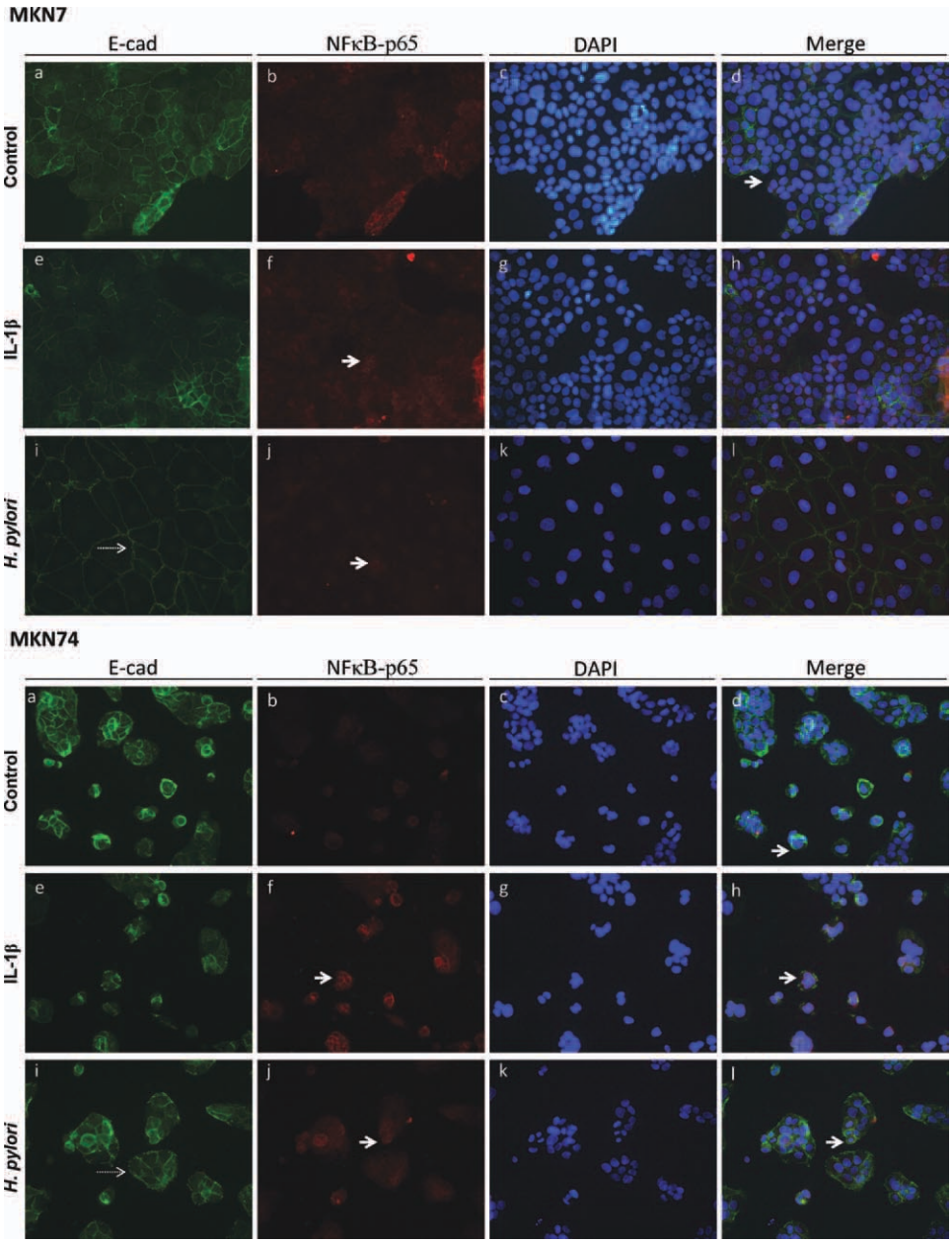

Figure 5. Immunofluorescence analysis of E-cadherin (E-cad) and nuclear factor κB (NFκB)-p65 subunit expression in gastric cancer cells treated with interleukin-1β (IL-1β) or cocultured with *H. pylori* is shown. Translocation of NFκB-p65 subunit from cytoplasm into the nucleus indicated by stronger fluorescent signal was observed in cells with IL-1β or *H. pylori* treatment as indicated by solid right arrow in the representative images. Cells infected with *H. pylori* showed patterns of destabilization in epithelial cell adherence, with the presence of internalized vesicles indicated by arrowhead with dashed connector. Nuclear morphology is shown with DAPI staining (in blue).

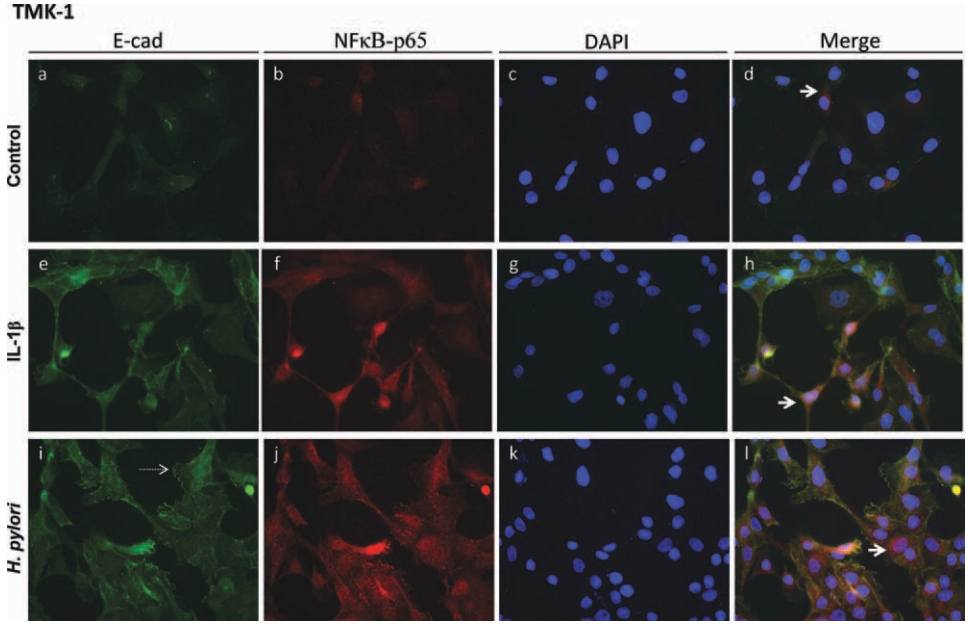

Figure 5. (*Continued*).

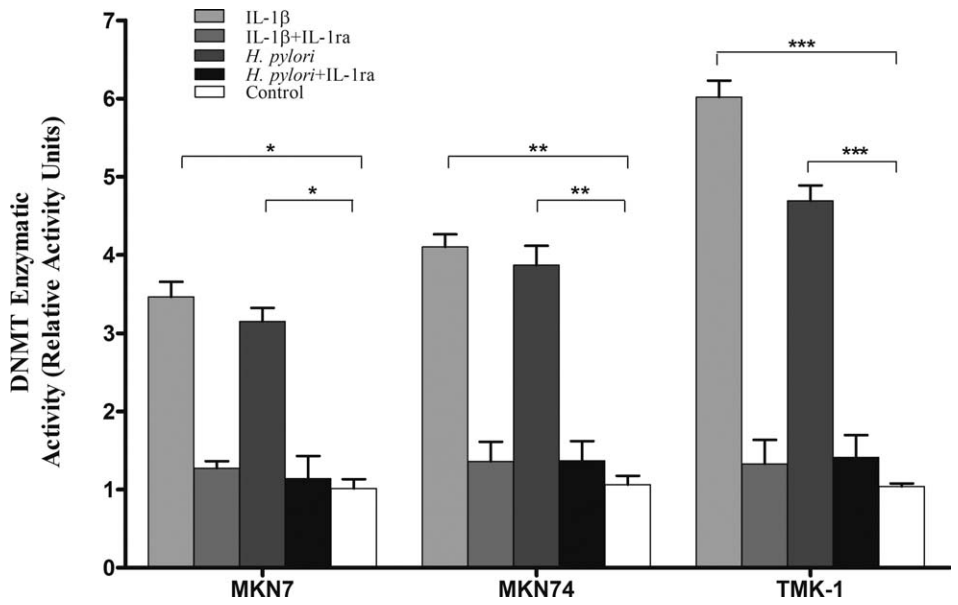

Figure 6. *H. pylori* infection and interleukin-1β (IL-1β) treatment stimulated total DNA methyltransferase (DNMT) activity in the 3 gastric cancer cell lines. MKN7, MKN74, and TMK-1 cells were pretreated with or without IL-1 receptor antagonist (IL-1ra) and were challenged with *H. pylori* or IL-1β as described in Materials and Methods. Total DNA methyltransferase enzymatic activity in treated and control cells were determined by the commercial kit as described. Data represent the mean ± standard error of the mean derived from 2 separate experiments with triplicate determination of total DNMT activity, and data are presented with reference to the control cells without treatment. ***$P < .0001$, **$P < .005$, and *$P < .01$.

mainly because of transcription activation by NFκB-p65 subunit. Thus, we performed immunofluorescence analysis for the translocation of NFκB-p50/65 subunits and found that both NFκB-p50 and NFκB-p65 migrate from cytoplasm into the nucleus upon IL-1β treatment or coculture with *H. pylori*, suggesting that NFκB-p50/65 subunits were translocated into the nucleus to activate gene transcription. Consistent with the Western blot findings, the fluorescent intensity of NFκB-p65 subunit was significantly higher than that of NFκB-p50 subunit in

nucleus. These findings suggest that *H. pylori* infection-induced inflammatory gene expression was mainly mediated through the NFκB-p65 signaling pathway.

Promoter methylation of E-cad led to down-regulation of mRNA expression. To determine whether the differences observed in E-cad mRNA expression levels translated into significant alterations of protein expression pattern, we performed immunofluorescence analysis for expression of E-cad protein. No significant characteristic change in the fluorescence intensity of E-cad was detected in these 3 cell lines upon treatment. However, infection with *H. pylori* tends to destabilize epithelial cell adherence, causing internalization of E-cad and the formation of intracellular vesicles. These data suggest that destabilizing epithelial cell adherence is an important factor in the pathogenesis of *H. pylori*-mediated gastric diseases.

In the present work, we demonstrated a significant elevation of NO production in cells challenged with IL-1β or *H. pylori*. Administration of cells with the antagonist IL-1ra abolished the observed phenomena. Thus, it is possible that *H. pylori*-induced E-cad methylation is via IL-1β activation of NFκB cascade that results in NO production by iNOS activation. Another important finding in our study is the up-regulation of the total DNMT enzymatic activity in cells treated with IL-1β or cocultured with *H. pylori*, DNMTs are the final effectors to methylate DNA that may account for the observed induction of E-cad methylation. These findings corroborate previous studies on activation of DNMT via NO production.[23-25] We further confirmed these findings by treating TMK-1 cells with an iNOS inhibitor, namely Nw-nitro-L-arginine methyl ester; we found that NO and DNMT activity returned to basal levels, and no methylation of E-cad was detected (data not shown). Taken together, we demonstrated that *H. pylori*-induced DNA methylation is mediated by NO, and suggest that NO plays a causative role in *H. pylori*-associated gastric cancer. This also explains the clinical observation that *H. pylori* infection causes gastric inflammation and carcinogenesis, and gives clues on the mechanism by which NO contributes to tumorigenesis.

H. pylori infection stimulates iNOS expression in the invading macrophages as well as in the inflamed gastric epithelial cells.[9] This stimulation is under transcriptional control of both proinflammatory cytokines and lipopolysaccharides.[26] The use of immortalized cell lines will eliminate the local environment, which is an important component of iNOS, particularly infiltrating macrophages. In the present study, we observed that *H. pylori* infection activates NO/iNOS via IL-1β production because of an intrinsic effect on gastric cells. However, Katayama et al reported that *H. pylori* did not increase DNA methylation in gastric cancer cells in the absence of macrophages, indicating that induction of DNA methylation was through NO produced from the cocultured macrophages.[25] The discrepancy is likely to have been initiated by the difference in the bacterial factors and host factors. It has been reported that the difference in the growth rate and proliferation of gastric cancer cells and the use of different *H. pylori* strains may result in different strength and effectiveness of inflammatory response and thus affect the induction of DNA methylation. Our findings were further supported by a recent study showing that different *H. pylori* strains, including the *H. pylori*-strain SS1 used in this study, have a strong potential to induce DNA methylation, regardless of their cagA status.[27] Therefore, we believe NO production during inflammation is strongly related to the magnitude and chronicity of stimulation, which most likely cause different detrimental effects to the cells. Taken together, we have described here an initial assessment on the mechanistic role of IL-1β involved in methylation induction during the inflammatory processes triggered by *H. pylori* infection, and have outlined a potential role of NO in mediating DNA methylation that links inflammation to carcinogenesis. However, given the complex nature of inflammation, an additional in vivo study has already been performed in our center using animal models lacking a functional IL-1β system to further characterize the mechanistic role of IL-1β in the interaction between *H. pylori*-induced host inflammatory response and DNA methylation.

In this study, we focused on the study of E-cad methylation, as E-cad plays a pivotal role in gastric cancers, and its alteration is an early event in gastric carcinogenesis.[17,18] In addition, it is important to characterize any specific epigenetic alterations in gastric precursor lesions leading to malignant transformation because of the reversible nature of epigenetic changes. This is supported by our previous finding that methylation of E-cad in *H. pylori*-infected gastric mucosa can be reversed after successful eradication of *H. pylori* infection.[21] Collectively, DNA methylation is potentially reversible by eliminating the triggering agents and together with the eradication of *H. pylori* might immediately reduce the risk of cancer development. It is known that eradication of *H. pylori* may not completely abolish the risk of gastric cancer; however, eradication therapy can stop gastric inflammation and eliminate production of NO, and reactive oxygen species thereafter may reverse many genetic and epigenetic changes that *H. pylori* infection induces in the

stomach, and thus prevent the development of gastric cancer and/or its precursor lesions.[28]

In conclusion, this study provides novel mechanistic insights into the pathogenesis of *H. pylori*. NO overproduction as a result of IL-1β stimulation of NFκB transcriptional system may play an important role in *H. pylori*-induced epigenetic changes in many cancer-relevant genes. This possible pathway lays out the role of NO in epigenetic modification linking inflammation to carcinogenesis.

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The authors made no disclosures.

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