E-cadherin promoter hypermethylation induced by interleukin-1β treatment or *H. pylori* infection in human gastric cancer cell lines

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**Abstract**

Interleukin-1β is up-regulated in the presence of *Helicobacter pylori* infection. *H. pylori* infection was associated with E-cadherin methylation. In this study, we examined if IL-1β could induce promoter methylation of E-cadherin in human gastric cancer cell lines TMK-1, MKN-74 and MKN-7. Cells were treated with IL-1β (0.025, 0.1, 0.25, 1.0, 2.5 ng/mL) for 6, 12 and 24 h. Methylation status was determined by MSP and sequencing. The effects of IL-1β or *H. pylori* on the cells, and after blockade with interleukin-1 receptor antagonist (IL-1ra) were tested. Promoter methylation of E-cadherin was induced in all three cells treated with IL-1β or co-cultured with *H. pylori*. Treatment of IL-1ra could reverse the phenomena. Our study indicated that IL-1β is an important step in mediating E-cadherin methylation.

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1. Introduction

*Helicobacter pylori* (*H. pylori*) infection is a World Health Organization group 1 carcinogen for developing gastric cancer. The gastric mucosal levels of interleukin-1 beta (IL-1β) is up-regulated in specimens infected with *H. pylori* both cagA positive strains and cagA negative strains [1]. IL-1β polymorphisms with T/T and T/C genotypes enhance IL-1β production, and are associated with an increased risk of *H. pylori* induced hypochlorhydria [2] and gastric cancer [3]. However, the underlying molecular mechanisms that contribute to this increased risk of gastric cancer are unknown.

*Helicobacter pylori* induced CpG island methylation has been reported in non-neoplastic gastric mucosa [4]. E-cadherin is one of tumour-suppressor genes and its promoter hypermethylation plays an important role in gastric carcinogenesis. We...
observed that patients with gastric cancer and dyspeptic patients with *H. pylori* infection frequently methylate the CpG island in the promoter region of the E-cadherin gene [4]. In addition, eradication of *H. pylori* infection in dyspeptic patients was associated with disappearance of methylation at the E-cadherin gene in non-neoplastic gastric mucosa [5,6]. It has been reported that IL-1 can modulate CpG island methylation through activation of DNA methyltransferase and by repressing gene expression [7].

We postulated that *H. pylori* infection and IL-1α polymorphism, via the increased production of IL-1α, predispose to gastric carcinogenesis by CpG island methylation of tumor suppressor genes. Our previous report showing that patients with *H. pylori* infection and IL-1α-511 T/T polymorphism have increased risk of developing gastric cancer corrobosrates this hypothesis [8]. In the current study, we further test this hypothesis by studying the role of IL-1α in inducing E-cadherin methylation in cultured cell lines.

2. Materials and methods

2.1. Cell lines and cell culture

The human gastric cancer cell lines TMK-1, MKN74 and MKN7 were purchased from the Cell Resource Bank (Ibaraki, Japan). TMK-1 is derived from patient with diffuse type gastric cancer, and MKN-74 and MKN-7 are derived from patients with intestinal type of gastric cancers. All three cell lines have no E-cadherin gene mutation or promoter methylation [9]. There was also no report on silencing of the E-cadherin gene in these cell lines.

Cells were used between passages 8 and 20 and cultured in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum (FBS) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin and 2.0 g/L NaHCO₃ at 37°C in a humidified atmosphere with 5% CO₂.

2.2. Exposure to IL-1α and blockade of IL-1α

After reaching confluence, cells were trypsinized and diluted in RPMI 1640 medium containing 10% FBS to a concentration of 5 × 10⁶ cells/ml and plated onto 6-well culture plates. After cultured for 24 h, cells were incubated with fresh serum-free medium (controls) or containing recombinant human interleukin-1β (rhIL-1β) (R&D Systems, Minneapolis, MN) (0.0252–5 ng/ml) [7,10,11]. In further experiments, the cells were treated with IL-1β alone or after a 60-min pretreatment with human interleukin-1 receptor antagonist (R&D Systems, Minneapolis, MN) (IL-1ra) at 10–100 ng/ml or co-cultured with *H. pylori* as described below.

2.3. Bacterial strain and conditions

*Helicobacter pylori* Sydney strain 1 (*H. pylori* SS1) were used. This strain has the cagA+ and vacA s2-m2 genotype. *H. pylori* was cultured on 7% horse blood agar plates (Columbia agar base, Oxoid) in microaerophilic conditions, and harvested between 48 and 72 h, resuspended in sterile phosphate buffered saline (PBS), and counted by absorbance at 660 nm (1 OD₆₆₀nm = 1 × 10⁸ colony forming units/ml).

Live *H. pylori* (at *H. pylori*/cell ratios of 0, 1/1, 5/1, 10/1, 50/1 and 100/1) were added to 6-well cell culture plates. The cells were co-cultured with *H. pylori* at 37°C in a humidified atmosphere before being collected. The co-cultures were also collected after a 60-min pretreatment with 20 ng/ml IL-1ra. The medium was harvested after culture and stored at −70°C.

2.4. Genomic DNA isolation and bisulfite modification

Genomic DNA was isolated from cells by High Pure PCR Template Preparation Kit according to the manufacturer’s instructions. DNA modified by the CpGenome DNA Modification kit (Chemicon International, Inc., CA, USA). Briefly, 1 μg of genomic DNA from each sample was denatured with 3 mol/L NaOH at 37°C for 10 min, followed by incubation with sodium bisulfate (pH 5.0) at 50°C for 16 h. Unmethylated cytosine residues are converted to uracil, whereas methylated cytosine residues remain unchanged. Modified DNA was recovered by ethanol precipitation and resuspended in PCR-grade water.

2.5. Methylation-specific polymerase chain reaction (MSP)

MSP distinguishes unmethylated from methylated alleles of a given gene based on sequence alterations produced by bisulfite treatment of DNA and subsequent PCR using primers specific to either methylated or unmethylated DNA. One-microliter aliquots of bisulfite-modified DNA were used as templates for PCR reactions. The PCR mixture contained 1 × PCR buffer (16.6 mM ammonium sulfate, 67 mM Tris [pH 8.8], 6.7 mM MgCl₂, and 10 mM 2-mercaptoethanol), 10 pM of each primer, 0.2 mM of each deoxynucleoside triphosphate (dNTP) and 1 U of Hot start Taq polymerase. The primers sequences specific for promoter methylated or unmethylated at E-cadherin and PCR condition have been described in previous report [12]. The methylated DNA known in previous study [5] was used as a positive control for methylation, and water was used as a negative control. PCR products...
were electrophoresed on 2% agarose gel, stained with ethidium bromide, and visualized under a UV illuminator. All tests were performed in duplicate.

2.6. DNA-sequencing analysis

These MSP products were excised from gels and purified (QIAquick Kits). They were further sequenced to determine the methylation status by invitrogen.

2.7. Statistical analysis

Where appropriate, data were analyzed using the Student’s t-test. A value of \( P < 0.05 \) was considered significant.

3. Results

3.1. Effect of IL-1\( \beta \) on E-cadherin promoter methylation

Gastric cancer cell lines TMK-1, MKN74 and MKN7 were incubated in the presence of different concentrations of IL-1\( \beta \) (0.025, 0.1, 0.25, 1.0 and 2.5 ng/ml) for 6, 12 and 24 h, and the promoter methylation of E-cadherin was estimated by MSP. Methylation of E-cadherin was absent in all cell lines before treatment but present after incubating with IL-1\( \beta \) (Fig. 1). Methylation of E-cadherin began to be detected at different dilutions of IL-1\( \beta \) and after different treatment time periods: TMK-1 cell line at 2.5 ng/ml for 6 h, MKN-74 cell line at 0.025 ng/ml for 6 h and MKN-7 cell line at 2.5 ng/ml for 24 h. Sequencing analysis showed cytosine–guanosine (CpG) sites within E-cadherin promoter region were partially methylated (Fig. 2).

3.2. Effect of IL-1ra on IL-1\( \beta \)-stimulated E-cadherin methylation

At highest dose (2.5 ng/ml) of IL-1\( \beta \) used in this study, methylation of E-cadherin was detected in all three cell lines. IL-1ra, an IL-1\( \beta \) inhibitor, blocked IL-1\( \beta \)-induced E-cadherin methylation. Cell lines were treated with 2.5 ng/ml of IL-1\( \beta \), with or without a 60-min pretreatment in the presence of plain medium or medium containing IL-1ra (10, 20, 50, 100 ng/ml). IL-1ra, as low as 20 ng/ml, blocked the effect of IL-1\( \beta \) compared to the control groups (Fig. 3) in all three cell lines.

3.3. Influence of H. pylori on E-cadherin methylation and IL-1ra blockade

To determine the effect of H. pylori on E-cadherin methylation, the gastric cancer cell lines TMK-1, MKN74 and MKN7 were co-cultured with H. pylori SS1 at a H. pylori/cell ratio of 1/1, 5/1, 10/1, 50/1 or 100/1 for 2–4 d. MSP analysis showed promoter methylation of E-cadherin was detected when TMK-1 cells were co-cultured with H. pylori at a H. pylori/cells ratio of 10–50/1 for 2 d, MKN-74 cells at a ratio of 5–10/1 for 3 d and MKN-7 cells at a ratio of 100/1 for 4 d, respectively (Fig. 4A). E-cadherin Methylation was not observed when cells were co-cultured with H. pylori after exposure to IL-1ra at 20 ng/ml for 1 h (Fig. 4B).

4. Discussion

Our studies showed that both IL-1\( \beta \) and H. pylori induced methylation of E-cadherin in gastric cancer cell lines in vitro and the methylation was blocked...
with the treatment of IL-1ra. We also found that different cell lines had different sensitivity when exposure to IL-1β.

We have previously demonstrated that IL-1B polymorphisms that correlate with higher levels of IL-1β increase the risks of gastric cancer in the presence of *H. pylori* infection [8]. However, the mechanisms are complicated and still uncertain. The present study demonstrates the role of IL-1β in inducing E-cadherin methylation and that *H. pylori* mediates E-cadherin methylation through IL-1β as well. Many studies have showed that IL-1β plays an important role in the pathogenesis of tumor[13,14]. The findings in this study were in line with that of previous study [7] and provided a new insight into the interaction between the pro-inflammatory cytokine and tumour-suppressor genes.

In addition, we observed that IL-1ra at an appropriate concentration could block the effect of IL-1β on methylation of E-cadherin. IL-1ra is a naturally occurring antagonist of the Type 1 interleukin-1 receptor (IL-1R1) and has been found useful in the reversal of IL-1 mediated effects in several pathological settings. Evans et al. found that endogenous IL-1ra has inhibitory effect on IL-1 activation or signaling and these concentrations are more than 100-fold lower than required for exogenous application of IL-1ra[15]. Thus, we speculate that differential expression of IL-1 system and in particular changes in the IL-1ra /IL-1β ratio during *H. pylori* infection, may contribute to the pleiotropic effect of IL-1β and determine why some individuals infected with *H. pylori* develop gastric cancer while others do not.

The importance of the E-cadherin gene in gastric carcinogenesis has been well demonstrated [16–18]. We observed in our previous studies that methylation of the E-cadherin promoter was associated with gastric cancer and that *H. pylori* infection was associated with E-cadherin methylation at the gastric mucosa in dyspeptic subjects without gastric cancer.

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The current study further showed in vitro that *H. pylori* infection was associated with *E*-cadherin methylation. We also used IL-1ra blockade to demonstrate that promoter methylation of *E*-cadherin observed in response to *H. pylori* is caused largely by IL-1β signaling.

However, the underlying mechanism of the effect of IL-1β on methylation in our present study will require further study. Veluthakal et al. found that IL-1β appears to have differential regulatory effects on two classes of protein-methylating enzymes in the pancreatic β cells. The lamin-B carboxyl methyltransferase was significantly increased in the nuclear fraction derived from IL-1β treated cells [19]. In contrast, the C terminal leucine methyltransferase activity appears to be significantly reduced by IL-1β treatment [20]. Hmadecha et al. further showed that IL-1β activated DNA methyltransferase via nitric oxide (NO) production [7]. NO is a gaseous free radical produced intracellularly by nitric oxide synthase (NOS)-mediated oxidation of arginine. *H. pylori* infection has been shown to be associated with up-regulation of inducible nitric oxide synthase (iNOS) both in vivo and in vitro [21,22]. Increased iNOS activity has been observed in patients with chronic gastritis and gastric adenocarcinoma patients [23]. Therefore, we hypothesize that *H. pylori* induces *E*-cadherin methylation through the action of IL-1β mediated by NO production.

The *H. pylori* strain SS1 was used in our present study. This strain is much used as the standard mouse-adapted strain for experimental infection. *H. pylori* SS1 failed to induce IL-8 secretion in gastric epithelial cells because of the likely absence of a functional of the cag pathogenicity island (cag PAI) [24]. However, it has been proven to be an excellent strain for use in the mouse colonization model. Studies showed that an increased expression of IL-1β and iNOS in SS1 strain-infected mice or cells were observed [25,26]. Kundu et al. further demonstrated that cag PAI is not the sole factor for the induction of pro-inflammatory cytokines [27].

We had also analyzed methylation at other genes in IL-1β or *H. pylori*-treated gastric cancer lines TMK-1, MKN74 and MKN7. These genes are involved in cell cycle regulation (p16), DNA repair or protection (*hMLH1*, MGMT), and apoptosis (DAP kinase). However, promoter methylation was detected in these genes in one or three cell lines before any treatment (data were not shown here). Hence no conclusion can be drawn. As we have shown before that cancer cells with *E*-cadherin methylation were only a minor subclone, which
was picked up by the sensitive MSP. It is hard to demonstrate the concordance between E-cadherin methylation and diminished E-cadherin expression [5].

Nonetheless, our study demonstrates that IL-1β plays an important role in E-cadherin methylation. We can preliminarily conclude from this study that Helicobacter pylori induces methylation of E-cadherin through the action of IL-1β. Further studies on the downstream effect of IL-1β on methylation are needed.

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References


