Activation of Intercellular Adhesion Molecule 1 Expression by Helicobacter pylori Is Regulated by NF-κB in Gastric Epithelial Cancer Cells

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Interactions between leukocytes and epithelial cells may play a key role in Helicobacter pylori-associated gastric mucosal inflammation. This process is mediated by various cell adhesion molecules. The present study examined the molecular mechanisms leading to H. pylori-induced epithelial cell intercellular adhesion molecule-1 (ICAM-1; also called CD54) expression. Coculture of epithelial cells with cytokinin-associated gene pathogenicity island-positive (cag PAI+) H. pylori strains, but not with a cag PAI− strain or H. pylori culture supernatants, resulted in upregulation of steady-state mRNA levels and cell surface expression of ICAM-1. Coculture with H. pylori induced an increase in luciferase activity in cells which were transfected with a luciferase reporter gene linked to the 5′-flanking region of the ICAM-1 gene. H. pylori activated the ICAM-1 promoter via the NF-κB binding site. An inducible nuclear protein complex bound to the ICAM-1 NF-κB site and was identified as the NF-κB p50–p65 heterodimer. H. pylori induced the degradation of IκB-α, a major cytoplasmic inhibitor of NF-κB, and stimulated the expression of IκB-α mRNA. Pretreatment of epithelial cells with pyrrolidine dithiocarbamate, which blocks NF-κB activation, inhibited H. pylori-induced ICAM-1 expression. THP-1 macrophagic cells, peripheral blood mononuclear cells, and purified neutrophils adhered to H. pylori-infected epithelial cells to a greater extent than to uninfected cells. These results show that H. pylori directly induces expression of ICAM-1 on gastric epithelial cells in an NF-κB-dependent manner that may support leukocyte attachment during inflammation.

Several lines of evidence implicate Helicobacter pylori in the pathogenesis of active chronic gastritis, peptic ulcer disease, gastric carcinoma, and gastric B-cell lymphoma, even though most H. pylori infections are asymptomatic (7, 29, 31). Although the exact mechanisms by which H. pylori infection results in gastroduodenal pathology are unclear, current opinion holds that H. pylori can induce an acute inflammatory reaction consisting of leukocyte infiltration and epithelium damage (4, 14). However, because H. pylori generally adheres to gastric epithelial cells without invading the epithelium, it is presumed that an interaction between bacteria and host epithelial cells may initiate H. pylori-induced inflammation. Because of the strong inflammatory response to H. pylori infections, the role of inflammatory cytokines was investigated. It was shown that mucosal biopsy specimens from patients with H. pylori infections contain significantly elevated levels of interleukin-1β (IL-1β), IL-6, tumor necrosis factor alpha (TNF-α), and IL-8 compared to those in specimens from uninfected individuals (9, 11, 13, 30). Exposure of gastric epithelial cell lines to H. pylori induced the secretion of a potent leukocyte chemotactic and activating factor, IL-8 (19, 32). The association of IL-8 with H. pylori infection was further suggested by elevated levels of IL-8 in gastric epithelial cells in vivo (9), in tissue homogenates of mucosal biopsy specimens (13, 27), and in in vitro-cultured biopsy specimens from patients with H. pylori-associated gastritis and peptic ulcers (30).

Cell-cell interactions play an important and probably central role in a large number of immunological processes in physiological and pathological conditions. These interactions are at least partially mediated by various cell adhesion molecules. Expression of cell adhesion molecules on gastric epithelial cells may participate in leukocyte homing and epithelial-cell adhesion in H. pylori-associated mucosal inflammation. In the context of H. pylori infection, the production of chemotaxic cytokines and cell adhesion molecules could provide a means of recruiting and retaining inflammatory cells within the gastric epithelial layer, contributing to H. pylori-mediated tissue injury.

In this study, we show that gastric epithelial cells have the ability to initiate changes associated with inflammation through the upregulation of intercellular adhesion molecule-1 (ICAM-1) after infection with H. pylori. Furthermore, we identify the molecular mechanism of ICAM-1 gene activation following H. pylori infection in gastric epithelial cell cultures. These data support the theory that the gastric epithelium plays an active role in initiating inflammation as part of the host response to H. pylori infection.

MATERIALS AND METHODS

Bacterial strains. The H. pylori strain ATCC 49503 was used in most of this study. Other clinical strains (OHPC0001, OHPC0002, and OHPC0003), isolated from chronic gastritis patients, were kind gifts of T. Kitahora (Ohkura Hospital, Tokyo, Japan). The presence of the cag pathogenicity island (PAI) and vacA in these strains was determined by PCR using specific sets of primers (1, 37). H. pylori strains were recovered from frozen stocks by seeding them on a blood agar plate (Mueller-Hinton II agar with 7% horse blood) at 37°C for 3 days under...
microaerophilic conditions (10%, O2 and 10% CO2) generated with Anaeropack OHPC0002, which totally lacked the ability to induce ICAM-1 expression (Fig. 2). Strain vacA expression. All isolates were positive for vacA expression. Since recent studies indicated that expression of vacA expression.

H. pylori induces ICAM-1 expression in human gastric epithelial cell lines. We first examined the capacity of several gastric epithelial cell lines to express ICAM-1 upon coculture with live H. pylori. AGS and KATO III cells constitutively expressed cell surface ICAM-1 and ICAM-1 mRNA, whereas MKN45 cells did not (Fig. 1A and B; also data not shown). Coculture with H. pylori significantly enhanced steady-state levels of ICAM-1 mRNA in MKN45 cells and cell surface expression of ICAM-1 by these cells (Fig. 1A and B). Similar results were also obtained using AGS and KATO III cells (data not shown). Since the H. pylori-induced ratio of enhancement of ICAM-1 expression was greater on MKN45 cells than on AGS- and KATO III cells, we next examined the dose dependency of H. pylori-induced ICAM-1 expression on MKN45 cells. The cell surface expression of ICAM-1 was significantly increased after the addition of 0.5 H. pylori organism/cell (Fig. 1C). The highest level of ICAM-1 expression was observed after infection with 50 H. pylori organisms/cell (Fig. 1C).

Flow cytometry studies indicated that MKN45 cells started to express a significant amount of ICAM-1 6 h after the initiation of coculture with the bacteria (Fig. 1B). ICAM-1 mRNA levels clearly increased 2 h after H. pylori infection, reached maximal levels at 6 h, and remained elevated at least 24 h following infection (Fig. 1A). H. pylori culture supernatants failed to induce ICAM-1 mRNA or cell surface expression on MKN45 cells (Fig. 1B and data not shown).

**RESULTS**

*H. pylori strains differ in their abilities to induce ICAM-1 expression.* Since recent studies indicated that expression of multiple genes in the *cag* PAI is necessary for cytokine production by gastric epithelial cells in vitro (6, 35), we examined the abilities of several *H. pylori* strains to induce ICAM-1 expression. All isolates were positive for *vacA* (1) irrespective of ability to induce ICAM-1 expression (Fig. 2). Strain OHP00002, which totally lacked the *cag* PAI, induced ICAM-1 mRNA and cell surface expression at levels consid-
erably lower than those of strains ATCC 49503, OHPC0001, and OHPC0003, containing the entire cag PAI (37) (Fig. 2). These results suggest that the *H. pylori* cag PAI may play an important role in the induction of ICAM-1 expression, which parallels a previously reported observation on IL-8 expression (6, 35).

**Activation of the ICAM-1 promoter by *H. pylori**. To confirm that *H. pylori*-induced ICAM-1 upregulation occurred by activating gene transcription, the ICAM-1 5′-flanking region was analyzed with promoter/reporter gene constructs. MKN45 cells were transiently transfected with a plasmid, pBHluc1.3, which contains 1,344 bp of the ICAM-1 upstream region (−1,353 to −9 relative to the start of transcription), directing expression of the firefly luciferase reporter gene, and the transfected cells were then stimulated with *H. pylori*. Stimulation of MKN45 cells with *H. pylori* resulted in a 6.6-fold increase in luciferase activity, whereas the negative-control vector, pBHlucOL1, showed only low background activity (Fig. 3B). Strains OHPC0001 and OHPC0003 induced ICAM-1 promoter activity to similar levels. However, strain OHPC0002, devoid of cag PAI, and *H. pylori* ATCC 49503 culture supernatants were not found to increase ICAM-1 promoter activity (data not shown). These results indicated that the interaction with *H. pylori* induces ICAM-1 expression on MKN45 cells at the level of transcription.

To locate the cis-regulatory element(s) present within the 5′-flanking sequence of the ICAM-1 promoter that confers *H. pylori* responsiveness, we transiently transfected a series of pBHluc1.3 mutants with 5′ deletions of various lengths (the largest mutant beginning with bp −941 and the smallest beginning with bp −136) into MKN45 cells (Fig. 3A). High levels of induction were observed with the reporter constructs containing ICAM-1 5′-flanking sequence starting with position −1,353 (pBHluc1.3) to position −353 (construct D). However, further deletion to −136 (construct E) resulted in a complete loss of *H. pylori*-induced promoter activity. It was apparent that the ICAM-1 5′-flanking sequence between −353 and −136 was required for the promoter to respond to *H. pylori*. To further investigate this *H. pylori*-responsive region, the sequence spanning bp −445 to −289 was cloned into construct E, resulting in construct P. This was not induced by *H. pylori*. Therefore, we concluded that the region of the ICAM-1 promoter most critical for *H. pylori* induction in gastric epithelial cells was located between −289 and −136.

Sequence analysis of the *H. pylori*-responsive region between −289 and −136 revealed potential binding sites for five transcription factors, AP-1 (−284 to −278), Sp1 (−206 to −201), C/EBP (−199 to −196), Ets (−153 to −150), and NF-κB (−187 to −178). Hou et al. (18) and Ledebur and Parks (25) demonstrated that TNF-α-induced activation of the ICAM-1...
promoter required the NF-κB site. In order to determine the functional importance of the NF-κB site, transient transfections with mutant ICAM-1 promoter/luciferase reporter gene constructs indicated that the NF-κB site is essential for activation by H. pylori in MKN45 cells. To determine whether the increase in ICAM-1 expression seen in H. pylori-stimulated gastric epithelial cells was related to an alteration of NF-κB DNA binding activity, nuclear extracts from gastric epithelial cells were prepared, and EMSA was performed using the ICAM-1 promoter NF-κB element as a probe. Figure 4A shows the results for MKN45 cell nuclear extracts. Control MKN45 cell nuclear extracts contained little activated NF-κB (Fig. 4A, lane 1). A single κB binding complex was observed in MKN45 cells after stimulation with H. pylori (Fig. 4A). Infection of KATO III and AGS cells with H. pylori resulted in NF-κB activation similar to that observed in MKN45 cells (data not shown). NF-κB activation was first evident at 30 min (Fig. 4A, lane 3) and appeared to be maximal at 1 h after H. pylori infection (Fig. 4A, lane 4). The levels of active NF-κB in nuclear extracts prepared from MKN45 cells after 2 h of exposure to H. pylori culture supernatants were similar to those in the control (data not shown). The specificity of this inducible κB-binding factor was confirmed by competition analysis with unlabeled oligonucleotides containing the ICAM-1 NF-κB site, the IL-2R α gene NF-κB site, or an ICAM-1 NF-κB site with a point mutation. NF-κB proteins appeared to mediate this H. pylori-induced binding, since unlabeled wild-type ICAM-1 (Fig. 4B, lane 3) or IL-2R α κB (lane 4), but not a mutant κB oligonucleotide (lane 5), effectively competed for binding to the NF-κB complex.

To identify the subunit composition of the NF-κB DNA binding complex, antibodies to NF-κB family members p65, c-Rel, p50, and p52 were preincubated with the nuclear extracts from MKN45 cells and used in supershift analysis (Fig. 4C). Anti-p65 specifically recognized the stimulated κB-DNA binding complex, resulting in supershifted complexes (Fig. 4C, lane 4). Preincubation with anti-c-Rel or anti-p52 did not affect the κB binding complex (Fig. 4C, lanes 5 and 6), but preincubation with anti-p50 resulted in the formation of a partially supershifted complex (Fig. 4C, lane 3). These results suggest that in MKN45 cells, an NF-κB p65–p50 heterodimer, and possibly a p65 homodimer, is rapidly induced in response to H. pylori infection.

The NF-κB site is sufficient to mediate cag PAI + strain-induced gene activation. Since it has been previously shown that cag PAI + strains induce significantly more IL-8 mRNA and protein than do cag PAI – strains (8, 10, 32), we sought to ascertain whether the difference between the abilities of different H. pylori strains to induce ICAM-1 expression was related to their abilities to activate NF-κB (Fig. 5A). NF-κB DNA binding activity was markedly induced by cag PAI + H. pylori strains (Fig. 5A, lanes 2, 3, and 5) compared to the cag PAI – strain (lane 4).

To further determine whether the NF-κB site was sufficient for activation by H. pylori, a reporter construct driven by an NF-κB element was compared (Fig. 5B). The construct κB-LUC, which contains five tandem repeats of the NF-κB site, was markedly stimulated by cag PAI + H. pylori strains, whereas infection of MKN45 cells with a cag PAI – strain resulted in only a slight increase in reporter activity (Fig. 5B). These results demonstrate that the NF-κB site is sufficient for cag PAI-specific activation of H. pylori-induced ICAM-1 gene expression.

Degradation of IκB-α by H. pylori. Since signal-induced proteolytic degradation of IκB-α precedes the appearance of NF-κB DNA binding activity, we determined whether IκB-α was degraded in MKN45 cells that were stimulated by H. pylori. Kinetic analysis of H. pylori-induced degradation of IκB-α in

FIG. 2. ICAM-1 induction on MKN45 cells by cag PAI + and cag PAI – H. pylori strains. (A) cag PAI + H. pylori strains show induced ICAM-1 mRNA expression in MKN45 cells compared with a cag PAI – H. pylori strain. Total RNA was extracted from cells cocultured with H. pylori for 6 h and used for Northern blot analysis. Equal loading was assessed by hybridization of stripped blots with a probe for GAPDH. (B) Cell surface expression of ICAM-1 on MKN45 cells was determined by fluorescence-activated cell sorter analysis. MKN45 cells were cultured for 24 h in culture medium (control) or in the presence of H. pylori. Representative results are shown as means ± standard deviations calculated from the results of three independent experiments.
induced with *H. pylori*. (B) The normalized relative luciferase activities corresponding to each construct transiently expressed in MKN45 cells that were left untreated or induced with *H. pylori* are shown. The activity of cells transfected with pBHlucOL1 without further treatment was designated 1. Average stimulated values were divided by average unstimulated values to give the induction ratios reported. Representative results are shown as means ± standard deviations (SD) calculated from the results of three independent experiments. (C) The ICAM-1 promoter activity in MKN45 cells after 3 h of culture (Fig. 6A).

**Induction of IκB-α mRNA expression in MKN45 cells by *H. pylori***. It is possible that *H. pylori* suppressed the transcription of the IκB-α gene. It has been reported that transcription of the IκB-α gene is regulated by the NF-κB binding site in the promoter and is activated by NF-κB p65 or c-Rel as part of a negative feedback loop (24). To examine whether *H. pylori* induces IκB-α, we carried out Northern analysis on IκB-α mRNA from MKN45 cells stimulated with *H. pylori*. MKN45 cells were cultured with *H. pylori* or in medium alone, and total RNA was extracted at 1, 2, 6, and 24 h. Induction of IκB-α mRNA by *H. pylori* was observed at 1 h and was maximal at 2 h, decreasing thereafter (Fig. 6B). These results clearly indicate that the decrease in IκB-α protein induced by *H. pylori* resulted not from suppression of transcription but from modulation of posttranscriptional events, probably at the protein level. Cumulatively, these results indicate that *H. pylori* activates MKN45 cells, as reflected by a loss of IκB-α protein and upregulation of its mRNA.

**Inhibition of NF-κB activation reduces ICAM-1 expression in response to *H. pylori* infection.** PDTC potently inhibits NF-κB activation and/or NF-κB interaction with its upstream regulatory binding site, thereby preventing NF-κB-mediated transcriptional activation (5, 21, 28, 38). We used PDTC to show a link between NF-κB activation and upregulation of ICAM-1 expression in *H. pylori*-infected MKN45 cells. As shown in Fig. 7, PDTC treatment markedly inhibited *H. pylori*-induced ICAM-1 expression in *H. pylori*-infected MKN45 cells. As shown in Fig. 8A, *H. pylori* significantly increased the adhesion of THP-1 cells, PBMCs, and purified neutrophils to MKN45 cells (11.8-, 5.8-, and 5.8-fold, respectively) compared with adhesion to MKN45 cells cultured in medium alone. Neutrophils were treated with an anti-CD18 MAb, an anti-CD54 MAb, or a control IgG1 before addition to *H. pylori*-infected MKN45 cells, and after 30 min, the adherence of the leukocytes was measured. As shown in Fig. 8B, the anti-CD18 MAb and the anti-CD54 MAb significantly suppressed the transcription of the ICAM-1 promoter activity in MKN45 cells that were left untreated or induced with *H. pylori* are shown. The activity of cells transfected with pBHlucOL1 without further treatment was designated 1. Average stimulated values were divided by average unstimulated values to give the induction ratios reported. Representative results are shown as means ± standard deviations (SD) calculated from the results of three independent experiments. (C) The ICAM-1 promoter activity in MKN45 cells after 3 h of culture (Fig. 6A).

**H. pylori** infection of epithelial cells increases leukocyte adhesion. MKN45 cells were infected for 6 h with *H. pylori*. THP-1 cells, PBMCs, or purified neutrophils were used as leukocytes for adhesion experiments. The cells were fluorescently labeled and added to infected or uninfected 6-h-*H. pylori*-infected MKN45 cells, and after 30 min, the adherence of the leukocytes was measured. As shown in Fig. 8A, *H. pylori* significantly increased the adhesion of THP-1 cells, PBMCs, and purified neutrophils to MKN45 cells (11.8-, 5.8-, and 5.8-fold, respectively) compared with adhesion to MKN45 cells cultured in medium alone. Neutrophils were treated with an anti-CD18 MAb, an anti-CD54 MAb, or a control IgG1 before addition to *H. pylori*-infected MKN45 cells, and the amount of adhesive cells was measured under these conditions. As shown in Fig. 8B, the anti-CD18 MAb and the anti-CD54 MAb significantly suppressed the transcription of the ICAM-1 promoter activity in MKN45 cells that were left untreated or induced with *H. pylori* are shown. The activity of cells transfected with pBHlucOL1 without further treatment was designated 1. Average stimulated values were divided by average unstimulated values to give the induction ratios reported. Representative results are shown as means ± standard deviations (SD) calculated from the results of three independent experiments. (C) The ICAM-1 promoter activity in MKN45 cells after 3 h of culture (Fig. 6A).

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FIG. 4. *H. pylori* induces a prominent NF-κB binding complex in MKN45 cells. (A) The time course of NF-κB activation in MKN45 cells infected with *H. pylori* was evaluated using EMSA. (B) Sequence specificity of NF-κB binding activity. The NF-κB probe was incubated with nuclear extracts from unstimulated (lane 1) or *H. pylori*-stimulated MKN45 cells (lanes 2 to 5) without (lane 2) or with a 100-fold excess of cold wild-type probes (lanes 3 and 4) or a 100-fold excess of mutant probe (lane 5). (C) Anti-p65 and anti-p50 sera recognize the NF-κB binding complex. The NF-κB probe was incubated with nuclear extracts from MKN45 cells that were left unstimulated (lane 1) or cocultured with *H. pylori* (lanes 2 to 6). The nuclear extracts were preincubated for 45 min with antibodies (Ab) against p50 (lane 3), p65 (lane 4), c-Rel (lane 5), or p52 (lane 6). The arrow indicates the location of the induced NF-κB binding complex.

decreased the adhesion of neutrophils to *H. pylori*-infected MKN45 cells, whereas the control IgG1 did not.

**DISCUSSION**

ICAM-1 is one of several cell adhesion molecules that belong to the immunoglobulin superfamily and serve as ligands for the β2 integrins, lymphocyte function-associated antigen-1 (LFA-1, also referred to as CD11a/CD18), and Mac-1 (CD11b/CD18). In contrast to LFA-1, which is constitutively expressed on leukocytes, ICAM-1 is an inducible cell surface glycoprotein expressed at a low level on a wide variety of cells, including leukocytes, vascular endothelium, fibroblasts, and certain epithelial cells. However, ICAM-1 expression is dramatically increased at sites of inflammation, providing important means of regulating cell-cell interactions and thereby presumably inflammatory responses. The function and indeed the importance of leukocyte adhesion in the generation and maintenance of inflammation have been demonstrated in numerous animal experimental systems using blocking MAbs directed against ICAM-1 as well as ICAM-1 knockout mice.

Researchers in several laboratories have demonstrated the expression of ICAM-1 in chronic gastritis associated with *H. pylori* in vivo (12, 16, 17). However, the mechanism whereby *H. pylori* induces the expression of ICAM-1 remains largely unknown. This study was therefore designed to evaluate ICAM-1 expression on gastric epithelial cells exposed to *H. pylori* in vitro.

First, we observed that *H. pylori* stimulated significant increases in steady-state mRNA levels and cell surface expression of ICAM-1 in gastric epithelial cells. Next, we investigated the molecular mechanisms by which ICAM-1 gene expression is regulated upon the exposure of gastric epithelial cells to *H. pylori* strains. TNF-α- and gamma interferon (IFN-γ)-induced activation of ICAM-1 have been reported to require different transcription factors. Hou et al. (18) and Ledebur and Parks (25) demonstrated that TNF-α-induced activation of the ICAM-1 promoter required a variant NF-κB site, 178 to 187 nucleotides upstream of the transcription start site. In contrast, Look and coworkers (26) demonstrated that the IFN-γ response element located at nucleotides −76 to −66 of the ICAM-1 5′-flanking region conferred IFN-γ responsiveness. The IFN-γ-induced DNA binding complex contained STAT1α (18). The data presented here indicate that the NF-κB binding sequence in the ICAM-1 promoter is required for *H. pylori*-induced ICAM-1 upregulation, whereas the IFN-γ response element was not essential.

*cap* PAI⁺ strains, unlike *cap* PAI⁻ strains, provoke potentially damaging inflammatory responses in infected host tissue and induce the synthesis of IL-8 in gastric biopsy specimens and cultured cells (8, 10, 32). Our study shows that the NF-κB p50–p65 heterodimer is activated in gastric epithelial cells in *H. pylori*-infected MKN45 cells, whereas the control IgG1 did not.
response to PAI+ strains of \textit{H. pylori}. Compared to a cag PAI- \textit{H. pylori} strain, the more-virulent cag PAI+ strains showed an enhanced ability to induce NF-κB binding activity, paralleling observations that increased ICAM-1 induction in MKN45 cells was cag PAI+ strain specific. Along these lines, increased IL-8 expression in epithelial cells in response to cag PAI+ \textit{H. pylori} strains may result from increased NF-κB binding to the IL-8 promoter (33). \textit{H. pylori} infection of the gastric epithelium, therefore, utilizes similar mechanisms to regulate the orchestrated production of ICAM-1 and IL-8.

Like many other NF-κB activators, \textit{H. pylori} induced the degradation of IκB-α in gastric epithelial cells. Degradation of IκB-α in \textit{H. pylori}-induced epithelial cells was associated with translocation of NF-κB and increased levels of IκB-α mRNA. Activated NF-κB was evident within 30 min of bacterial infection and was pronounced at 1 h. These findings are consistent with the NF-κB activation observed in \textit{H. pylori}-infected epi.
thelial cells before ICAM-1 mRNA levels are increased, followed in turn by increased cell surface ICAM-1 expression. Furthermore, the blocking of NF-κB activation by PDTC markedly inhibited cell surface ICAM-1 expression after H. pylori infection. These findings suggest that NF-κB activation may be a necessary prerequisite for increased epithelial-cell ICAM-1 expression in response to bacterial infection by H. pylori.

Our experiments using cell-free H. pylori culture supernatants suggest that the activation of ICAM-1 expression in gastric epithelial cells might not be mediated by a soluble product released from the bacterium. Other investigators have reported that direct contact with live H. pylori is indispensable for IL-8 production by gastric epithelial cells (1, 22). Expression of ICAM-1 was upregulated in vitro by inflammatory cytokines, IL-8 production by gastric epithelial cells (1, 22). Expression of ICAM-1 expression in response to bacterial infection by H. pylori may be a necessary prerequisite for increased epithelial-cell ICAM-1 mRNA levels before ICAM-1 protein expression. The blocking of NF-κB by PDTC inhibited the production of ICAM-1 mRNA and protein in gastric epithelial cells. Blocking the NF-κB activation could not induce the production of ICAM-1 mRNA and protein in gastric epithelial cells. Blocking the NF-κB activation could not induce the production of ICAM-1 mRNA and protein in gastric epithelial cells.

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RETRACTION

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