Helicobacter pylori Activates Calpain via Toll-Like Receptor 2 To Disrupt Adherens Junctions in Human Gastric Epithelial Cells[▽]

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Helicobacter pylori is a risk factor for the development of gastritis, gastroduodenal ulcers, and gastric adenocarcinoma. *H. pylori*-induced disruption of epithelial adherens junctions (AJs) is thought to promote the development of severe disease; however, the mechanisms whereby *H. pylori* alters AJ structure remain incompletely understood. The present study demonstrates that *H. pylori* infection in human patients is associated with elevated serum levels of an 80-kDa E-cadherin ectodomain, whose presence is independent of the presence of serum antibodies against CagA. *In vitro*, a heat-labile *H. pylori* surface component activates the host protease calpain in human gastric MKN45 cells independently of the virulence factors CagA and VacA. *H. pylori*-induced calpain activation results in cleavage of E-cadherin to produce a 100-kDa truncated form and induce relocalization of E-cadherin and β -catenin. Stimulation of MKN45 cells with the toll-like receptor 2 (TLR2) ligand P3C activated calpain and disrupted E-cadherin and β -catenin in a pattern similar to that induced by *H. pylori*. Inhibition of TLR2 prevented *H. pylori*-induced calpain activation and AJ disassembly. Together, these findings identify a novel pathway whereby *H. pylori* activates calpain via TLR2 to disrupt gastric epithelial AJ structure.

Helicobacter pylori is a Gram-negative bacterium that colonizes the gastric mucosa of over one-half of the human population. In the absence of treatment, infection with *H. pylori* will persist for the lifetime of the host. All individuals infected with *H. pylori* develop chronic atrophic gastritis, and in a subset of hosts infection progresses to the development of peptic ulcers, gastric adenocarcinoma, or mucosa-associated lymphoid tissue (MALT) lymphoma (19, 24, 25, 44). Several *H. pylori* virulence factors are associated with the development of more-severe disease; indeed, strains expressing the translocated protein CagA or the secreted vacuolating cytotoxin VacA pose a greater risk for the development of ulcerative disease and adenocarcinoma (2, 27, 28, 32). Despite these findings, the precise mechanisms determining the clinical outcome of infection remain incompletely understood.

Intercellular attachment of epithelial cells is maintained via tight junctions and adherens junctions (AJs) and is critical for gastrointestinal epithelial homeostasis; tight junctions help regulate cell polarity and epithelial barrier function, and AJs maintain tissue architecture and cell polarity and suppress cellular proliferation and migration (41). AJs are composed of the transmembrane protein E-cadherin, which forms loose, homotypic, Ca^{2+} -dependent interactions with E-cadherin molecules of neighboring cells. Intracellularly, distinct sites on the cytoplasmic tail of E-cadherin interact with proteins of the catenin family, including β -catenin and γ -catenin, which are linked to the actin cytoskeleton via α -catenin (30). β -Catenin has at least

two distinct cellular functions: membrane-localized β -catenin serves as a structural component of the AJ, and a cytosolic pool of β -catenin acts as a downstream effector in the Wnt signaling pathway. Activation of Wnt signaling induces a series of events that culminate in nuclear translocation of β -catenin and binding of the lymphoid enhancer factor (LEF)/T cell factor (TCF) transcription factors to activate targeted transcription of genes involved in apoptosis, proliferation, and carcinogenesis, including *MMP-7*, *c-myc*, *cyclin D*, and *COX-2* (38). Downregulation of E-cadherin is frequently associated with increased invasive growth and metastasis (6), and cleavage of the extracellular domain of E-cadherin produces a fragment that promotes cellular invasion *in vitro* (22, 35). This E-cadherin ectodomain is present in elevated levels in the serum and urine of cancer patients (20). Consistent with these roles, abnormalities in β -catenin and E-cadherin expression or distribution have a well-established association with the development of gastric cancer (18, 40).

Recent studies have shown that *H. pylori* can alter AJ integrity, leading to pathology. A carcinogenic strain of *H. pylori* induced CagA-dependent β -catenin activation and tumor formation in a gerbil model of disease (14). Moreover, *in vitro* transfection experiments have shown that CagA interacts with E-cadherin, leading to β -catenin activation and subsequent transdifferentiation from a gastric to an intestinal epithelial phenotype (29). Other studies have shown CagA-independent deregulation of the AJ complex by *H. pylori* (1, 45). Nevertheless, the mechanisms underlying *H. pylori*-induced AJ disruption remain unclear. A better understanding of the cellular pathways by which this bacterium disrupts epithelial AJs may shed light on mechanisms of *H. pylori*-related disease.

μ -Calpain (calpain-1) and m-calpain (calpain-2) are ubiqui-

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tously expressed, Ca^{2+} -dependent cysteine proteases that regulate many cellular processes, including cell motility (16), survival (37), apoptosis (23), and inflammation (46). Both E-cadherin and β -catenin are calpain substrates, and proteolytic targeting of these proteins by calpain deregulates β -catenin signaling (33, 34). In airway epithelia, toll-like receptor 2 (TLR2)-induced calcium fluxes have been shown to mediate bacterially induced calpain activation, leading to cleavage of E-cadherin (8). *H. pylori* lipopolysaccharide is a TLR2 agonist, and TLR2 expression is strongly upregulated on the apical surface of the gastric mucosa of *H. pylori*-infected patients and *H. pylori*-challenged cultured gastric epithelial cells (36, 43, 52). In light of these findings, we hypothesized that calpain may mediate *H. pylori*-induced AJ defects and aimed to assess the roles of TLR2 and calpain in *H. pylori*-induced AJ disruption using human samples and an *in vitro* gastric epithelial model. Our findings reveal a novel mechanism of *H. pylori* pathogenesis wherein *H. pylori* activates calpain via TLR2 to disrupt gastric epithelial E-cadherin and β -catenin.

MATERIALS AND METHODS

Assessment of *H. pylori* infection and CagA status of human subjects and serum E-cadherin enzyme immunoassay. Serum samples were collected from adult (≥ 18 years of age) patients in the Calgary Health Region and catchment area between November 2003 and November 2005. Stool samples were collected, and stool antigens for *H. pylori* were assessed using a commercially available immunoassay (Immunocard STAT HpSA; Meridian Diagnostic Inc., Cincinnati, OH). Serum anti-CagA antibody levels were quantified using an anti-CagA human IgG enzyme-linked immunosorbent assay (ELISA) kit in accordance with the manufacturer's instructions (Genesis Diagnostics, Littleport, Cambridgeshire, United Kingdom). Serum samples were diluted 1:200 in sample diluents and assayed in duplicate alongside positive and negative controls provided by the manufacturer. Based on the value measured in the negative-control serum (~ 590 U/ml), and in agreement with the manufacturer's instructions, samples with 600 U/ml or less were considered negative for anti-CagA IgG. Serum levels of soluble E-cadherin were also quantified using a commercially available ELISA (Zymed, San Francisco, CA) in accordance with the manufacturer's instructions.

Cell culture. MKN45 human gastric adenocarcinoma cells (Health Science Research Resources Bank, Osaka, Japan) were cultured in RPMI 1640 medium (Sigma, St. Louis, MO). SCBN cells were grown in Dulbecco's modified Eagle's medium (Sigma) containing 100 g/ml streptomycin, 100 U/ml penicillin, 0.8 mg/ml tylosin, and 200 mM L-glutamine (all from Sigma). SCBN cells, originally obtained from a human patient, have subsequently been characterized to have a canine genotype and a nontumorigenic intestinal epithelial cell phenotype (3); they form polarized, confluent monolayers with functional tight junctions, express adherens junction proteins that localize to the cell-cell contact, exhibit permeability changes in response to microbial stimuli, and form tight attachments with *H. pylori* strain SS1 (5, 12). Media for both cell lines were supplemented with heat-inactivated fetal bovine serum (HI-FBS; 10% for MKN45 and 5% for SCBN) and maintained at 37°C with 5% CO_2 in 96% humidity. Culture medium was replenished every 2 to 3 days. Cells were passaged every 6 to 8 days with 2 $\times$ trypsin-EDTA (Sigma). Cells were grown to confluence in Lab-Tek chamber slides (Nalge-Nunc International, Naperville, IL) for immunocytochemistry experiments and on tissue culture-treated 6-well plates or 96-well plates (Costar, Cambridge, MA) for immunoblotting and activity assays, respectively. MKN45 cells were used between passages 25 and 35, and SCBN cells were used between passages 21 and 25.

Bacterial culture and infection of cells. The wild-type (WT) *H. pylori* strain 60190 from humans (CagA⁺ VacA⁺; American Type Culture Collection, Manassas, VA) and its isogenic CagA and VacA mutant strains (originally created by Richard Peek Jr., Nashville, TN, and kindly provided to us by Nicola Jones, Hospital for Sick Children, Toronto, ON, Canada) were grown from a 10% glycerol frozen stock in 10% HI-FBS brucella broth (Becton Dickinson) on a shaker (150 rpm) for 48 h at 37°C in microaerophilic conditions (5% O_2 , 10% CO_2 , 85% N_2) generated by CampyGen packets (Oxoid, Nepean, Ontario, Canada). Log phase cultures were centrifuged at 1,000 $\times$ g, resuspended in cell

culture medium, and added apically to cell monolayers at a multiplicity of infection of 100:1 for up to 24 h. For some experiments, heat-killed bacteria were prepared by boiling a broth culture for 2 min or bacteria were killed by exposure to antibiotics (100 g/ml streptomycin, 100 U/ml penicillin, and 0.8 mg/ml tylosin; all from Sigma). Inocula were tested for numbers of live bacteria by serial dilution and plating on Columbia blood agar (Sigma). All experiments were conducted in serum-free cell culture medium.

Calpain activity assay. MKN45 monolayers were loaded with 20 μM *t*-butoxy-carbonyl-L-leucyl-L-methionine amide (CMAC; Molecular Probes, Eugene, OR), a calpain-specific, membrane-permeable fluorogenic substrate. In serum-free conditions, cells were stimulated with the appropriate treatments and fluorescence was quantified over a 24-h period using a Spectramax M2e microplate reader (Molecular Devices, Sunnydale, CA).

Reagents, inhibitors, and antibodies. The following antibodies were used for immunoblotting or immunocytochemistry: mouse monoclonal anti-E-cadherin (DECMA-1 clone), rabbit polyclonal anti-pan-cadherin, and mouse monoclonal anti- β -catenin (all from Sigma) and mouse monoclonal anti-pan-actin (Thermo Fisher Scientific, Waltham, MA). The selective calpain inhibitor PD150606 (EMD, Darmstadt, Germany) was used at a concentration of 50 μM 30 min prior to infection (13). TL2.1, a neutralizing monoclonal antibody against TLR2 (10 $\mu\text{g}/\text{ml}$; eBioscience, San Diego, CA) was used 1 h prior to infection. Inhibitors and neutralizing antibodies remained in the medium throughout the infection. For some experiments, MKN45 cells were incubated with the TLR2 agonist P3C (15 $\mu\text{g}/\text{ml}$; Sigma) for up to 6 h.

Whole-cell protein extraction. MKN45 or SCBN monolayers grown in 6-well plates were washed twice with phosphate-buffered saline (PBS) and lysed for 30 min on ice with 150 μl of radioimmunoprecipitation assay (RIPA) buffer (1 $\times$ PBS, 1% Igepal CA-630, 0.5% sodium deoxycholate, and 0.1% SDS; all from Sigma) containing a protease inhibitor tablet (Complete-Mini; Roche Diagnostics, Laval, QC, Canada). Lysates were sonicated and centrifuged at 10,000 $\times$ g for 10 min. Supernatants were collected, and protein concentrations were quantified using a Bradford assay (Bio-Rad, Hercules, CA). Protein concentrations were normalized to 5 mg/ml, and samples were diluted in 2 $\times$ electrophoresis buffer at a ratio of 1:1 and boiled for 3 min. Samples were stored at -20°C until use.

Western blotting. Samples were separated by SDS-PAGE (10 to 13%) and transferred to nitrocellulose membranes (Whatman, Buckinghamshire, England). Membranes were blocked for 1 h in 5% nonfat dry milk or 5% bovine serum albumin (BSA) in Tris-buffered saline (TBS) plus 0.1% Tween (TBS-T) and probed with primary antibodies overnight at 4°C. Membranes were then washed 3 times for 5 min in TBS-T and incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (1:1,000 dilution; Cell Signaling Technology) for 1 h at room temperature. Bands were visualized using the ECL-plus chemiluminescence detection system (GE Healthcare, Pittsburgh, PA), and band density was quantified using a Canon CanoScan 4400F scanner and Image J densitometry software (<http://rsbweb.nih.gov/ij/>). To assess protein loading, membranes were stripped in 0.5 M acetic acid for 30 min and reprobed for pan-actin.

Immunocytochemistry. Cell monolayers grown on chamber slides were washed twice with sterile PBS and fixed/permeabilized in absolute methanol for 30 min at 4°C. Nonspecific binding was blocked with HI-FBS for 15 min at room temperature. Cells were incubated in primary antibodies for 1 h at 37°C, washed twice with PBS, and incubated with the appropriate Alexa Fluor-conjugated secondary antibody (1:2,000 dilution; Molecular Probes) for 1 h at 37°C. Nuclei were counterstained for 5 min using 1 μM Hoechst 33258 (Invitrogen). Slides were then mounted using Aqua Poly/Mount (Polysciences, Warrington, PA) and visualized using a Leica DMR fluorescence microscope. Photomicrographs were taken using a Retiga 2000R camera (QImaging, Surrey, BC, Canada) and Velocity 4.3.1 photoimaging software (Improvision, Waltham, MA).

Statistical analysis. Parametric data are expressed as means $\pm$ standard errors of the means (SEM). Comparisons of parametric data were made using the Student *t* test or analysis of variance (ANOVA), as appropriate. *Post hoc* analysis was performed with the Student-Newman-Keuls test. Nonparametric data from samples from human subjects are expressed as medians and represented pictorially as scatter plots, with whiskers representing upper and lower quartiles. Nonparametric data were compared using the Mann-Whitney test. For all statistical tests, significance was established at *P* values of <0.05 .

RESULTS

***H. pylori*-infected patients have elevated serum levels of soluble E-cadherin.** Elevated levels of a soluble, 80-kDa form of

FIG. 1. Soluble E-cadherin is elevated in the serum of *H. pylori*-positive patients. Serum from *H. pylori*-positive patients and uninfected controls was assessed for levels of the soluble 80-kDa ectodomain using ELISA. *H. pylori*-positive patients ($n = 8$) had significantly elevated levels of the soluble 80-kDa E-cadherin ectodomain compared to the median of *H. pylori*-negative controls ($n = 8$) (A). Levels of soluble E-cadherin do not differ between *H. pylori*-positive patients with anti-CagA serum immunoreactivity ($n = 3$) and CagA antibody-negative, *H. pylori*-positive individuals ($n = 5$) (B). Data are expressed as scatter plots with the center lines representing medians and the short lines representing upper and lower quartiles. *, $P < 0.05$ compared to the median for uninfected or CagA antibody-negative controls.

E-cadherin have been detected in the circulation and urine of cancer patients (20), and this soluble fragment has been shown to promote epithelial cell invasion *in vitro* (31). To investigate the effects of *H. pylori* infection on adherens junction integrity in human patients, we conducted an ELISA for this soluble E-cadherin ectodomain on serum from patients infected with *H. pylori* and uninfected control subjects. *H. pylori*-positive patients had significantly higher serum levels of soluble E-cadherin than controls (Fig. 1A). In order to investigate whether generation of soluble E-cadherin was related to the *H. pylori* virulence factor CagA, we conducted an ELISA for anti-CagA IgG as a marker of individuals infected with CagA-positive strains. We then assessed whether this increase in soluble E-cadherin was associated with the presence of anti-CagA antibodies. Of the *H. pylori*-positive patients, there was no significant difference in the levels of soluble E-cadherin in patients who were positive for anti-CagA serum IgG compared to those who were not (Fig. 1B).

***H. pylori* induces internalization of β -catenin and disruption and cleavage of E-cadherin in epithelial monolayers.** To investigate the effects of *H. pylori* on adherens junction structure *in vitro*, we challenged MKN45 human gastric epithelial cells with *H. pylori* strain 60190 (a Cag pathogenicity island [PAI]⁺ vacA⁺ human isolate) for 24 h. To assess reproducibility, we repeated these key experiments in an additional *in vitro* model using a nontumorigenic canine intestinal epithelial cell line (SCBN) challenged with *H. pylori* strain SS1. This model has been used previously to illustrate *H. pylori*-induced disruption of tight junctional proteins (12). SCBN cells have the advantage of being nontumorigenic cells that form polarized, confluent monolayers with membrane localization of both E-cadherin and β -catenin. In both models, Western blotting revealed that a 24-h *H. pylori* challenge induced cleavage of E-cadherin to produce a 100-kDa fragment (Fig. 2A), which was associated with redistribution of E-cadherin away from the cell-cell contact as shown by immunocytochemistry (Fig. 2B). Similarly, a 24-h infection with *H. pylori* induced translocation of β -catenin from the membrane to an intracellular compartment (Fig. 2B).

***H. pylori* activates μ -calpain in MKN45 monolayers.** Given that previous reports have shown the 100-kDa E-cadherin fragment to be the result of cleavage by μ -calpain (33), we then assessed whether calpain is activated in *H. pylori*-challenged epithelia. MKN45 cells coincubated with *H. pylori* 60190 for 24 h were immunoblotted for μ -calpain using an antibody that detects both the full-length and cleaved, active forms of the enzyme. Challenge with *H. pylori* enhanced levels of the active 75-kDa form of μ -calpain (Fig. 3A). To confirm this finding, MKN45 cells were loaded with a cell-permeable fluorogenic calpain substrate (CMAC) and calpain activity was measured over time. These experiments revealed a significant time-dependent activation of calpain in cells challenged with *H. pylori* compared with sham-treated controls. Elevated calpain activity was detected as early as 12 h postinfection and was sustained until the end of the 24-h measurement period (Fig. 3B).

***H. pylori* activates calpain independently of CagA or VacA.** In order to assess whether this enhanced calpain activity was due to CagA or VacA, both well-characterized *H. pylori* virulence factors, MKN45 cells were challenged with isogenic CagA⁻ or VacA⁻ 60190 mutants and calpain activity was measured using the CMAC activity assay. Both the CagA⁻ and VacA⁻ strains enhanced calpain activity in MKN45 monolayers to the same extent and with the same kinetics as the WT strain (Fig. 3B).

Calpain is activated by a heat-labile *H. pylori* surface component. In an attempt to further characterize the potential *H. pylori* virulence determinant responsible for calpain activation, the CMAC calpain activity assay was performed on cells challenged with antibiotic-killed or boiled *H. pylori* to assess the requirement for live bacteria or a microbial protein. Antibiotic-killed bacteria increased calpain activity to the same extent as live *H. pylori*, whereas calpain activity in cells exposed to boiled cultures did not differ from that of sham-treated controls (Fig. 4). These data suggest that a proteinaceous *H. pylori* surface component is responsible for calpain activation.

Calpain disrupts adherens junctions during *H. pylori* challenge. To assess whether the observed AJ defects were mediated by *H. pylori*-induced calpain activation, MKN45 monolay-

FIG. 2. *H. pylori* induces adherens junction disruption in epithelial monolayers. MKN45 or SCBN monolayers were challenged with *H. pylori* 60190 or SS1, respectively, for 24 h and immunoblotted for E-cadherin (A) or immunostained for E-cadherin or β-catenin (B). *H. pylori* challenge induced cleavage of E-cadherin to produce a truncated 100-kDa form in both MKN45 and SCBN cell monolayers (A). *H. pylori*-infected monolayers demonstrated loss of E-cadherin from the cell-cell contact and a diffuse (MKN45) or punctate (SCBN) cytosolic accumulation of E-cadherin (B). *H. pylori* also induced internalization and a nuclear/perinuclear distribution of β-catenin (B). $n = 3$ per group. Con, control.

ers were pretreated with the selective calpain inhibitor PD150606 30 min prior to *H. pylori* challenge. Pretreatment with PD150606 reduced *H. pylori*-induced E-cadherin cleavage (Fig. 5A) and prevented its redistribution (Fig. 5B). Pretreatment with the calpain inhibitor also prevented *H. pylori*-induced β-catenin redistribution (Fig. 5B). Treatment with the inhibitor alone had no effect on these parameters.

FIG. 3. *H. pylori* activates μ-calpain independently of CagA and VacA. MKN45 monolayers challenged with *H. pylori* 60190 for 24 h had increased levels of the 75-kDa active form of μ-calpain (A) and showed significantly increased calpain activity over time, as assessed by measuring production of a fluorogenic calpain substrate (B). Isogenic 60190 CagA (ΔCagA) or VacA (ΔVacA) mutant strains increased calpain activity to the same level and with the same kinetics as WT 60190 (B). $n = 3$ to 8 per group. *, $P < 0.05$ compared to sham-treated controls.

***H. pylori* activates calpain via TLR2 to disrupt AJs.** We next investigated the mechanisms of *H. pylori*-induced calpain activation. Since calcium signaling downstream of TLR2 activation has been shown to activate calpain in airway epithelia (8), we hypothesized that *H. pylori* might disrupt AJs via TLR2-mediated calpain activation. To investigate the role of TLR2 in our model, MKN45 cells were challenged with the TLR2 agonist P3C for up to 6 h and calpain activity and AJ structure were assessed via calpain activity assay and immunocytochemistry, respectively. P3C significantly enhanced calpain activity in MKN45 cells 2 h posttreatment (Fig. 6A) and induced rearrangement of E-cadherin and β-catenin in a pattern similar to that induced by *H. pylori* (Fig. 6B). Furthermore, pretreatment with the calpain inhibitor PD150606 prevented this P3C-induced AJ protein rearrangement (Fig. 6B). We then assessed the role of TLR2 during *H. pylori* challenge; 1 h of pretreatment with a TLR2 neutralizing antibody prior to 24 h of in-

FIG. 4. Calpain is activated by a heat-labile *H. pylori* surface component. Treatment of MKN45 monolayers with boiled *H. pylori* 60190 failed to induce calpain activation, as assessed by measuring production of a fluorogenic calpain substrate, whereas antibiotic-killed 60190 bacteria were able to activate calpain to the same extent as live bacteria. $n = 8$ per group. *, $P < 0.05$ compared to sham-treated controls.

FIG. 5. Calpain mediates *H. pylori*-induced adherens junction defects. Pretreatment of MKN45 monolayers with the calpain inhibitor PD150606 (PD) decreased *H. pylori* 60190-induced production of a 100-kDa E-cadherin cleavage fragment (A) and prevented 60190-induced E-cadherin and β -catenin redistribution (B). Treatment with PD150606 alone had no effect on these parameters. Cells were infected with *H. pylori* for 24 h. *n* = 3 per group.

fection with *H. pylori* prevented calpain activation (Fig. 7A) and redistribution of E-cadherin and β -catenin (Fig. 7B). Treatment with the neutralizing antibody alone had no effect on these parameters. These data suggest that activation of TLR2 induces calpain-dependent AJ disruption in MKN45 gastric epithelial cells and that TLR2 activation is an upstream event in calpain-mediated AJ disruption during *H. pylori* infection.

FIG. 6. TLR2 activates calpain and induces calpain-dependent E-cadherin and β -catenin rearrangement in gastric epithelial monolayers. Treatment of MKN45 monolayers with the TLR2 agonist P3C significantly increases calpain activity at 2 h posttreatment (A) and induces membrane-to-cytosol translocation of E-cadherin and β -catenin 6 h posttreatment, which is preventable by pretreatment with the calpain inhibitor PD150606 (B). *n* = 3 per group.

FIG. 7. *H. pylori* activates μ -calpain via TLR2 to disrupt E-cadherin and β -catenin. Pretreatment of MKN45 monolayers with the TLR2 neutralizing antibody TL2.1 prevents *H. pylori*-induced generation of the active 75-kDa form of μ -calpain (A) and prevents *H. pylori*-induced rearrangement of E-cadherin and β -catenin (B). Cells were infected with *H. pylori* for 24 h. TL2.1 alone had no effect on E-cadherin or β -catenin distribution. $n = 3$ per group.

DISCUSSION

The mechanisms whereby *H. pylori* promotes carcinogenesis remain unclear. A growing body of evidence indicates that *H. pylori* can disrupt the structure and function of intercellular AJ complexes, a process that has a clear association with oncogenesis in many organs, including the stomach (9). In most cancers of epithelial origin, loss of E-cadherin is associated with acquisition of an invasive phenotype, and germ line mutations of E-cadherin are found in familial gastric cancer (18). β -Catenin is aberrantly activated in gastric cancer precursor lesions (40), suggesting that AJ modifications precede adenocarcinoma development. Studies using a gerbil-adapted strain of *H. pylori* in an *in vivo* model of disease have shown that β -catenin activation plays a role in gastric tumorigenesis (14), and AJ disruption has been linked to increased cell migration (10, 45) and cellular transdifferentiation from a gastric to an intestinal phenotype *in vitro* (29). However, despite advances in this area, the mechanisms whereby persistent *H. pylori* infection alters gastric epithelial AJs remain incompletely understood.

Our findings demonstrate the ability of *H. pylori* to alter AJ structure at several levels. First, we have shown that *H. pylori* infection is associated with enhanced serum levels of an 80-kDa "invasion promoter" fragment produced by cleavage of E-cadherin in its extracellular domain. Prior studies have shown that this fragment inhibits cadherin-based contacts in a paracrine fashion, thereby promoting cell invasion *in vitro* (11, 22, 31). Furthermore, serum levels of soluble E-cadherin have been identified as a prognostic indicator for gastric adenocarcinoma (4). Our findings corroborate those of a recent study showing that *H. pylori* can induce E-cadherin ectodomain shedding *in vitro* in an ADAM 10-dependent fashion (35). Furthermore, *H. pylori* induces expression of matrix metalloproteinase

7 (26, 47), a host protease that has been shown to cleave the extracellular domain of E-cadherin (11). Alternatively, a bacterial protease may be responsible for E-cadherin ectodomain shedding. Indeed, the colonic bacterium *Bacteroides fragilis* secretes *B. fragilis* toxin, a protease that induces E-cadherin ectodomain shedding (48, 49). Interestingly, a small study has documented a significantly increased prevalence of enterotoxigenic *B. fragilis* in the stools of colorectal cancer patients, which has led to the hypothesis that the proteolytic targeting of E-cadherin by *B. fragilis* toxin may contribute to the development of colorectal cancer via activation of β -catenin-dependent transcription (42, 49). Our study shows, for the first time, that E-cadherin ectodomain shedding is associated with *H. pylori* infection in human subjects.

H. pylori infections are consistently associated with a reduction in E-cadherin expression *in vivo*, which may be due to promoter hypermethylation. However, it is becoming clear that *H. pylori* can also have more-direct effects on the AJ. Indeed, *in vitro* *H. pylori* can rapidly disrupt the integrity of the AJ without affecting E-cadherin and β -catenin expression (10, 45). Our results also indicate that *H. pylori* disrupts AJs at an intracellular level via activation of the host cell protease calpain. Calpains are ubiquitously expressed, Ca^{2+} -dependent proteases that are activated by Ca^{2+} fluxes in the micromolar (μ -calpain) or millimolar (m-calpain) range (17). Calpains have been implicated in the control of a variety of cellular processes, including inflammation (46), cell migration (16), and cell survival (37), and m-calpain is upregulated in localized and metastatic prostate cancer tissue (33). Both μ - and m-calpain can cleave the cytoplasmic tail of E-cadherin between residues 782 and 787 to produce a truncated 100-kDa form that lacks the β -catenin binding domain (33). Calpain can also directly cleave β -catenin, resulting in its nuclear export and

activation of TCF-dependent transcription (34). In the present study, we describe a novel mechanism whereby *H. pylori* induces activation of epithelial calpain, which results in truncation of E-cadherin to its 100-kDa form and AJ disassembly, characterized by redistribution of both E-cadherin and β -catenin.

Infection with *H. pylori* enhances gastric epithelial expression of TLR2 *in vitro* and is associated with strongly upregulated apical epithelial expression of TLR2 in human gastric biopsy specimens (43). *H. pylori* lipopolysaccharide has been shown to activate epithelial TLR2 to stimulate cell proliferation and the production of inflammatory chemokines via induction of mitogen-activated protein kinase (MAPK) (52) and NF- κ B (36) signaling, respectively. Furthermore, a recent study using airway epithelial cells has shown that binding of TLR2 by its ligand can activate calpain via induction of phosphatidylinositol 3-kinase (PI3K)- and phospholipase C- γ (PLC- γ)-dependent Ca^{2+} release (7, 8). This, in turn, results in calpain-induced cleavage of E-cadherin and the tight junctional protein occludin and subsequent opening of intercellular junctions to facilitate the transepithelial migration of neutrophils (8). In the present study, stimulation of gastric epithelial cells with P3C, a TLR2 ligand, results in calpain activation and disruption of E-cadherin and β -catenin localization in a pattern similar to that induced by *H. pylori*. Furthermore, inhibition of TLR2 using a neutralizing antibody prevents *H. pylori*-induced calpain activation and AJ disassembly. Therefore, our data indicate that *H. pylori*-induced activation of calpain and subsequent AJ disruption depend on activation of epithelial TLR2. Future studies may offer corroborating evidence in support of the present TLR2 blocking studies by using genetic or other approaches.

Our data suggest that both shedding of the E-cadherin ectodomain and calpain-dependent AJ disassembly occur independently of CagA. Although previous studies have defined a role for CagA in AJ disruption (14, 15), *H. pylori* may decrease membrane levels of β -catenin in a Cag pathogenicity island (PAI)-independent manner (1). Similarly, both internalization of the cadherin/catenin complex and shedding of the E-cadherin ectodomain have been shown to occur independently of CagA *in vitro* (45). Interestingly, the basolaterally expressed protein β 1 integrin has been identified as the receptor for the type IV secretion system (T4SS), which is required to inject CagA into the host cell (21). These findings imply that Cag PAI-independent opening of the paracellular pathway must occur to allow contact of the T4SS with its receptor. Data from the present study suggest that this may occur via the activation of intracellular proteases by pattern recognition receptors.

Furthermore, our data indicate that *H. pylori*-induced calpain activation does not require VacA or live bacteria, but rather is dependent on a heat-labile *H. pylori* surface component. Indeed, a substantial proportion (approximately 4%) of the *H. pylori* genome is predicted to encode outer membrane proteins (39). Many of these proteins function as adhesins and enhance *H. pylori* pathogenesis. One such protein, OipA, is known to affect disease outcome and has been shown to mediate *H. pylori* adhesion to the epithelium and coregulate pro-inflammatory cytokine production (50, 51). Studies using a gerbil model of disease have shown that OipA augments the incidence and severity of *H. pylori*-induced dysplasia and ade-

nocarcinoma and is required for nuclear translocation of β -catenin (15). Future studies are needed to explore the hypothesis that OipA is the heat-labile surface component that activates TLR2 to effect calpain-dependent AJ disruption during *H. pylori* infection.

In conclusion, findings from the present study demonstrate that *H. pylori* induces disruption of AJ complexes at multiple levels. The results have elucidated a pathway whereby activation of TLR2 by *H. pylori* leads to activation of calpain to effect changes associated with carcinogenesis, including intracellular E-cadherin cleavage and β -catenin translocation. We propose that this mechanism of TLR/protease-mediated AJ disruption represents a CagA-independent early stage in a multifaceted pathogenic strategy to open the AJs. Given the well-established tumor-suppressive function of intact AJs, further research using *in vivo* models of disease is warranted to assess the relevance of TLR2/calpain-mediated AJ disruption in *H. pylori*-induced carcinogenesis.

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