Up-regulated Smad5 Mediates Apoptosis of Gastric Epithelial Cells Induced by Helicobacter pylori Infection*

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The gastric pathogen Helicobacter pylori activates epithelial cell signaling pathways, and its infection induces changes in the expression of several genes in infected human gastric tissues. Recent studies have indicated that the ability of H. pylori to regulate epithelial cell responses depends on the presence of an intact cag pathogenicity island (cagPAI). We investigated altered mRNA expression of gastric epithelial cells after infection with H. pylori, both cagPAI-positive and cagPAI-negative strains, by cDNA microarray, reverse transcription PCR, and Northern blot analysis. Our results indicated that cagPAI-positive H. pylori strains (ATCC 43504 and clinical isolated strains) significantly activated Smad5 mRNA expression of human gastric epithelial cell lines, RPMI 1640 containing 10% fetal calf serum under microaerophilic conditions as described above on a gyratory shaker at 170 rpm for 24–36 h to the plateau phase. The human gastric cell lines AGS, KATOIII, MKN28, and MKN45 were obtained from the Japanese Research Resources Bank (Tokyo, Japan) and were maintained in a complete medium consisting of RPMI 1640 medium supplemented with 10% fetal calf serum (Invitrogen) under microaerophilic conditions as described above on a gyratory shaker at 170 rpm for 24–36 h to the plateau phase. The human gastric cell lines AGS, KATOIII, MKN 28, MKN 45 were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan) and were maintained in a complete medium consisting of RPMI 1640 medium supplemented with 10% fetal calf serum (Invitrogen) under microaerophilic conditions as described above on a gyratory shaker at 170 rpm for 24–36 h to the plateau phase. The bacteria were then suspended in sterile phosphate-buffered saline. After centrifugation, the bacteria

EXPERIMENTAL PROCEDURES

Bacterial Strains and Cell Lines—Biopsy specimens were obtained from Japanese patients in Hokkaido University Hospital and were cultured on H. pylori-selective agar plates (Eiken Chemical Co., Ltd., Tokyo, Japan) under microaerophilic conditions (5% O2, 10% CO2, 85% N2, at 37 °C). Peptic ulceration and gastric cancer occur in some people with H. pylori infection, but the majority remain asymptomatic. Although differences among the degrees of gastric mucosal damage caused by different strains should be an important factor for development of various clinical outcomes, these strain differences do not provide a complete explanation for individual differences in H. pylori infection-induced gastric mucosal injury. Therefore, it is presumed that host responses also play an important role in the outcome of H. pylori infection, interacting with virulence factors and environmental factors. Recent studies have shown that H. pylori induces various cellular responses, proliferation, apoptosis (9), cytokine secretion (13). In this study, we investigated the altered gene expression of host cells infected with cagPAI-positive or cagPAI-negative H. pylori strain and the association between the altered gene expression and the cellular responses.

Helicobacter pylori is a human pathogen that infects the gastric mucosa and causes an inflammatory process leading to gastritis, gastric ulceration, duodenal ulceration, mucosa-associated lymphoid tissue lymphoma, and gastric cancer (1). The pathogenesis of gastroduodenal diseases caused by this bacterium is not well understood. Since the whole genome of H. pylori was sequenced in 1997, several putative virulence factors, including VacA (2), IceA, OipA (3), HrgA (4), lipopolysaccharide, and the neutrophil-activating protein (5), have been elucidated. The cag pathogenicity island (cagPAI), a complex of genes coding ~30 proteins, has been reported to be a major virulence factor of H. pylori. The cagPAI is acquired by horizontal transfer and is found in about 50–70% of H. pylori isolates in Western countries and in more than 90% of H. pylori isolates in Asian countries, including Japan (6, 7). This lesion codes for the type IV secretion machinery system forming a cylinder-like structure connected to epithelial cells (8). Many virulence gene products or other interactive proteins might be transferred into the host cells via this system. Peptic ulceration and gastric cancer occur in some people with H. pylori infection, but the majority remain asymptomatic. Although differences among the degrees of gastric mucosal damage caused by different strains should be an important factor for development of various clinical outcomes, these strain differences do not provide a complete explanation for individual differences in H. pylori infection-induced gastric mucosal injury. Therefore, it is presumed that host responses also play an important role in the outcome of H. pylori infection, interacting with virulence factors and environmental factors. Recent studies have shown that H. pylori induces various cellular responses, proliferation, apoptosis (9), cytokine secretion (13). In this study, we investigated the altered gene expression of host cells infected with cagPAI-positive or cagPAI-negative H. pylori strain and the association between the altered gene expression and the cellular responses.

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Co-culture of Epithelial Cells with H. pylori—Human gastric epithelial cell lines were cultured in RPMI 1640 containing 10% fetal calf serum without antibiotics and used at a final concentration of 5 × 10^6/ml. Bacterial suspensions were cultured at 37 °C in brain heart infusion broth containing 10% fetal calf serum under microaerophilic conditions as described above on a gyratory shaker at 170 rpm for 24–36 h to the plateau phase. The bacteria were then suspended in sterile phosphate-buffered saline. After centrifugation, the bacteria
were resuspended at a final concentration of 1×10^7 colony-forming units (cfu)/ml in RPMI 1640 supplemented with 10% fetal calf serum and used immediately. Gastric epithelial cells alone or cells with bacteria were cultured in tissue culture dishes (Falcon, Becton Dickinson) at 37 °C in a humidified incubator in an atmosphere of 95% air and 5% CO_2. The cells were washed with phosphate-buffered saline three times after 4, 8, 12, and 24 h. Total cellular RNA was extracted from the cells by using Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer’s instructions, and the amount was measured by absorbance at 260 nm.

cDNA Microarray Procedure—Poly(A) RNA was isolated from total cellular RNA (100 ng) using an MagExtractor (Toyobo, Tsuruga, Japan) according to the manufacturer’s instructions. Total cellular RNA was isolated in combination with oligo(dT) magnetic beads (in the kit), and then nonspecific substance was removed by washing. 2 μg of mRNA was reverse transcribed into cDNA by reverse transcriptase, ReverTraAce (Toyobo), in the presence of a CDNA synthesis primer. Biotin-labeled probes were generated by binding of biotin-16-deoxyuridine triphosphate during synthesis of cDNA. The human cDNA expression filters, human cancer filters (Toyobo) were prehybridized at 62 °C for 30 min in 20 ml of PerfectHyb solution (Toyobo). After denaturalization, cDNA probes were hybridized to the filters overnight at 62 °C. The membranes were washed three times with solution 1 (2× SSC and 0.1% SDS) and three times with solution 2 (0.1× SSC and 0.1% SDS) for 5 min at 62 °C. Specific signals on the filters were detected by using a chemiluminescence detection kit, Imaging High (Toyobo), according to the manufacturer’s instructions. CDP-Star was used as the chemiluminescent substrate. Images and quantitative data of gene expression levels were obtained using a Fluor-S Multimager system (Nippon Bio-Rad Laboratories, Tokyo, Japan) and quantified into intensity of signals by using ImaGene (BioDiscovery, Inc., Los Angeles, CA).

**RESULTS**

**Up-regulated Smad5 Mediates H. pylori-induced Apoptosis**

**Arabidopsis thaliana**

**Analysis of Apoptosis**—We used two methods to detect apoptosis of epithelial cells induced by *H. pylori* infection. After co-culture of AGS cells with *H. pylori* for 72 h, DNA was extracted from the control and treated groups using an apoptosis laddering detection kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Each DNA (5 μg) was electrophoresed on a 1% agarose gel containing 6.5% formaldehyde and then transferred onto a nylon membrane. A Smad5 probe was made from human Smad5 cDNA that corresponded to its whole coding region. Each probe was labeled with biotin using Biotin-16-DUTP (Roche Diagnostics, Tokyo, Japan). A human β-actin probe labeled with biotin was used as a positive control. The membrane was hybridized with the labeled probe for 20 h at 62 °C in PerfectHyb (Toyobo). After hybridization, it was washed three times with 2× SSC with 0.1% SDS for 10 min and washed three times with 0.1× SSC with 0.1% SDS for 10 min at 62 °C. Positive bands were detected by using a chemiluminescence detection kit (Imaging High, Toyobo), and CDP-Star was used as the chemiluminescent substrate according to the manufacturer’s instructions.

**mRNA Expression by RT-PCR**—First strand cDNA templates were synthesized from 2 μg of total RNA using ReverTraAce and a random primer (Toyobo) according to the manufacturer’s instructions. An aliquot (0.1 μl) of Taq DNA polymerase and deoxynucleoside triphosphates (Takara Shuzou Co., Ltd., Shiga, Japan) was mixed with 0.5 μl of 10× PerfectHyb solution and cDNA sample and each primer was used in a separate PCR reaction. The sense primer Smad5F (5'-CAACACACGCTTCTGTGTTCA-3') and Smad5R (5'-TTGA-CACAAACCACCAAGC-3') for Smad5 amplification. PCR was performed using a thermal cycler (Takara Shuzou) under the following conditions: an initial denaturation for 5 min at 94 °C; 30 s at 94 °C, 30 s at 55 °C, and 30 s at 72 °C; and a final extension at 72 °C for 5 min with the number of cycles at which the band intensity increased linearly with the amount of mRNA used. The PCR product was then run on a 1.5% agarose gel.

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**Interference of Smad5 mRNA**—Two 29-mer DNA oligonucleotides (siRNA oligonucleotide templates) with 21 nucleotides encoding the siRNA and 8 nucleotides complementary to the T7 promoter primer were chemically synthesized, desalted, and purified by reverse phase high pressure liquid chromatography. These sequences were subjected to a BLAST search (NCBI data base) to ensure that only one gene was targeted. Two 21-mer oligonucleotides (sense, 5'-AATACATCCCTGC-CGGTGGTA-3' and antisense, 5'-AATATCACCAGCCAGGGATGAA-3') encoding Smad5 had no homology to those of Smad1, 2, 3, 4, and 8 in a BLAST search. The two siRNA oligonucleotide templates were hybridized to a T7 promoter primer and were extended by the Klenow DNA polymerase. The sense and antisense siRNA templates were transcribed by T7 RNA polymerase and were hybridized to create double-stranded siRNA using a Silencer siRNA construction kit (Ambion). The control and *H. pylori*-treated cells were grown in 96-well plates, and cationic lipid-mediated transient transfections were carried out with 50 ng of siRNA/well using GeneSilencer siRNA transfection reagent (Gene Therapy Systems, San Diego, CA). After incubation at 37 °C for 24 h, Northern blot analysis was performed to assess the effectiveness of RNA interference, and quantitative analysis of apoptosis was carried out as described above.

**Statistics**—The data are presented as the means ± S.D. The differences were examined by analysis of variance, and *p* values <0.01 were considered significant.

**RESULTS**

**Smad5 Up-regulation in Gastric Epithelial Cell Lines**—We first examined changes in gastric cellular mRNA expression in response to co-culture with *H. pylori* (capPAI-positive, ATCC 43504 strain) at 8 and 24 h by cDNA microarrays in AGS cells. Eleven housekeeping genes were used as internal controls to correct the mRNA abundance. Although the majority of genes indicated only small differences, the expression level of Smad5 mRNA increased dramatically (Fig. 1), with the relative fold changes in density to housekeeping genes being 0.4, 22.4, and 21.9 at 0, 8, and 24 h, respectively. The expression of the other Smad family (Smad1, 2, 3, 4, and 8) mRNA including R-Smads were increased 0.6–1.3-fold after 24 h co-culture and were not significant. Northern blot analysis was carried out to confirm the overexpression of Smad5 mRNA. Total RNA was extracted from AGS cells treated with *H. pylori* and untreated AGS cells at 4, 8, 12, and 24 h. Northern blot analysis showed that...
**DISCUSSION**

The gastric pathogen *H. pylori* activates epithelial cell signaling pathways after infection. However, the exact signaling pathways are still unknown. The host immune response to *H. pylori* infection might be of importance with regard to the various clinical outcomes of infection by this organism. We now report that *H. pylori* can up-regulate the Smad5 expression of gastric epithelial cells and that the Smad5 up-regulation is involved in *H. pylori*-induced apoptosis of gastric epithelial cells. In addition, it was found that the presence of intact cagPAI is essential for Smad5-mediated apoptosis of epithelial cells.

We speculated that a paracrine or autocrine system of TGF-β and bone morphogenetic proteins (BMP) from infected *H. pylori* or AGS cells are involved in the up-regulation of Smad5 mRNA expression, the induction of apoptosis was completely inhibited in the quantitative apoptosis assay (Fig. 6).

### FIG. 3. *H. pylori* up-regulated Smad5 expression in other human gastric epithelial cell lines and in native gastric mucosa. a, total RNA was extracted from human gastric epithelial cell lines (KATOIII, MKN28, and MKN45) co-cultured with live *H. pylori* for the indicated time intervals, and the expression of Smad5 mRNA was analyzed by RT-PCR using the specific primers. Smad5 mRNA expressions were up-regulated in all of the tested cells. The fold change of density was indicated. β-actin was amplified as a control in parallel. b, total RNA was extracted from the five native gastric biopsy specimens from five patients infected with cagPAI-positive strains or the five native gastric biopsy specimens from five uninfected patients, and the expression of Smad5 mRNA was analyzed by RT-PCR using the specific primers. Smad5 mRNA were highly expressed in cagPAI-infected gastric mucosa. The fold change of density is indicated.

### FIG. 4. cagPAI-positive *H. pylori* up-regulated Smad5 expression of AGS cells by Northern blot analysis. Smad5 mRNA expression of AGS cells after co-cultured with cagPAI-negative (strains 42 and 273) and cagPAI-positive (strains 192, ATCC 43504, 912, 904, and 878) *H. pylori* strains at 30 h. a, cagPAI-positive *H. pylori* strains induced up-regulation of Smad5 significantly, compared with cagPAI-negative strains. The fold change of density was indicated. b, after RNA interference, the up-regulated Smad5 mRNA expressions induced by *H. pylori* infection were suppressed in cagPAI-positive strains. The β-actin probe was hybridized as a control.

### FIG. 5. Induction of Apoptosis

Induction of Apoptosis—DNA fragmentation was induced in AGS cells 72 h after having been co-cultured with cagPAI-negative (strains 42 and 273) and cagPAI-positive (strains 192, ATCC 43504, 912, 904, and 878) *H. pylori* strains at 30 h. a, cagPAI-positive *H. pylori* strains induced up-regulation of Smad5 significantly, compared with cagPAI-negative strains. The fold change of density is indicated. b, after RNA interference, the up-regulated Smad5 mRNA expressions induced by *H. pylori* infection were suppressed in cagPAI-positive strains. The β-actin probe was hybridized as a control.
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not shown). Although TGF-β1 and BMP in co-cultured supernatants from H. pylori-infected and uninfected AGS cells were measured by enzyme-linked immunosorbent assay, significant differences were not found (data not shown). Additionally, TGF-β1 mRNA and BMP mRNA were not up-regulated after co-culture with H. pylori in cDNA array experiments. Furthermore, because H. pylori itself did not possess TGF-β1 or BMP-like genes, which was examined by BLAST search (NCBI data base), it is unlikely that a paracrine or autocrine pathway of TGF-β or BMP from AGS infected with H. pylori or direct production of TGF-β or BMP from H. pylori is involved in up-regulation of Smad5 expression.

The cagPAI region encodes a novel H. pylori secretion system, type IV machinery (16), and this apparatus is essential for the induction of interleukin-8 via an NF-κB-dependent transcriptional process in human gastric cells (17, 18). It has recently been shown that CagA is injected from the attached H. pylori into host cells via the type IV machinery and that it forms a physical complex with SHP-2, the Src homology 2 domain-containing tyrosine phosphatase, in a phosphorylation-dependent manner and stimulates the phosphatase activity (11, 19). These findings suggest that protein or gene injection through the type IV machinery is a key mechanism for host-bacterial interaction induced by H. pylori infection. Consequently, it is not surprising that the transcriptional response of gastric epithelial cells is dependent on the presence of cagPAI. We therefore examined the Smad5 expression of AGS cells using cagPAI-positive and cagPAI-negative strains and that of the native gastric mucosa infected with H. pylori. Our results indicated that cagPAI-positive H. pylori strains were able to activate Smad5 mRNA expression and to induce apoptosis of the infected epithelial cells but that cagPAI-negative strains were not able to activate Smad5 mRNA expression or induce apoptosis. Although CagA is the only H. pylori protein known to translocate from the bacterium into the cell via the type IV secretion system, it can be assumed that transfer of unknown genes or gene products through the type IV machinery might be necessary for up-regulation of the Smad5 gene in host cells.

It has been reported that mutations in Smad4 played a significant role in the progression of colorectal tumors (20) and that a subset of families with juvenile polyposis had germ line mutations in the Smad4 gene and were at increased risk of developing gastrointestinal cancers (21). However, because there has been no report on Smad5 expression in gastrointestinal tract, the role of Smad5 in physiological or pathological status is not known.

Smad family proteins have molecular masses of about 42–65 kDa. Eight different Smads have been identified in mammals and can be classified into three subclasses, receptor-regulated Smads (R-Smads), common mediator Smads (Co-Smads), and inhibitory Smads (I-Smads) (22). Each member of the Smad family plays a different role in signaling pathways. R-Smads can be further subdivided into two subtypes, those phosphorylated after stimulation by TGF-β and BMP. Smad5 belongs to the latter group (23). Smad5 was isolated as dwarfin-C and was genetically implicated in TGF-β-like signaling pathways in Drosophila and Caenorhabditis elegans (24). Suzuki et al. (25) proposed that Smad5 directs the formation of the ventral mesoderm and epidermis in Xenopus embryos. In an antisense oligonucleotide study, Smad5 was shown to mediate the growth inhibitory effect in hematopoietic cells (26), and Yamamoto et al. (27) suggested that Smad5 inhibited myogenic differentiation. Furthermore, BMP actively mediated apoptosis in the embryonic limb (28), and BMP-2 also induced apoptosis in...
human myeloma cell lines, probably via up-regulation of R-Smads (Smads1, 5, and 8) (29). Many studies have demonstrated that _H. pylori_ induced apoptosis of gastric epithelial cells (30), suggesting that the up-regulated Smad5 mRNA expression might be involved in the apoptosis of gastric epithelial cells induced by _H. pylori_ infection.

We also confirmed that only cagPAI-positive _H. pylori_ strains were capable of inducing up-regulation of Smad5 mRNA as well as having apoptotic effects in human gastric cells. Although virulence factors, VacA, and lipopolysaccharide have been investigated as possible apoptosis-inducing factors (31), though virulence factors, VacA, and lipopolysaccharide have been investigated as possible apoptosis-inducing factors (31), the precise intracellular signaling mechanism of apoptosis induced by _H. pylori_ is still unknown. Our results indicated that Smad5 up-regulation might be related to the apoptosis induced by cagPAI-positive _H. pylori_ infection as one of the intracellular signaling molecules. We therefore compared the levels of _H. pylori_-induced apoptosis before and after suppression of Smad5 mRNA expression by RNA interference, and it was found that the induction of apoptosis was reduced to the background level after the interference. These observations suggest that Smad5 up-regulation is a key factor for _H. pylori_-induced apoptosis. In conclusion, _H. pylori_-up-regulates Smad5 expression through the presence of cagPAI encoding type IV secretion machinery, and up-regulated Smad5 induces apoptotic responses in infected gastric epithelial cells.

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