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Spermine Oxidase Mediates the Gastric Cancer Risk Associated With *Helicobacter pylori* CagA

Rupesh Chaturvedi^{*,†}, Mohammad Asim^{*,†}, Judith Romero-Gallo^{*}, Daniel P. Barry^{*,†}, Svea Hoge^{*,‡}, Thibaut de Sablet^{*,†}, Alberto G. Delgado^{*}, Lydia E. Wroblewski^{*}, M. Blanca Piazuolo^{*}, Fang Yan[¥], Dawn A. Israel^{*}, Robert A. Casero Jr.[#], Pelayo Correa^{*}, Alain P. Gobert^{*,†,‡}, D. Brent Polk^{¥,¶}, Richard M. Peek Jr.^{*,§,†}, and Keith T. Wilson^{*,§,†}

^{*}Division of Gastroenterology, Hepatology, and Nutrition, Department of Medicine, Vanderbilt University Medical Center, Nashville, TN

[§]Department of Cancer Biology, Vanderbilt University Medical Center, Nashville, TN

[†]Veterans Affairs Tennessee Valley Healthcare System, Nashville, TN

[‡]Department of General, Abdominal and Vascular Surgery, Otto-von-Guericke University, Magdeburg, Germany

[¥]Division of Gastroenterology, Department of Pediatrics, Vanderbilt University Medical Center, Nashville, TN

[¶]Department of Pediatrics, University of Southern California Keck School of Medicine, Los Angeles, CA

[#]Department of Oncology, Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University School of Medicine, Baltimore, MD

[‡]Institut National de la Recherche Agronomique, Unité de Microbiologie UR454, Saint-Genès-Champagnelle, France

Correspondence: Keith T. Wilson, M.D., Vanderbilt University School of Medicine, Division of Gastroenterology, Hepatology, and Nutrition, 1030C MRB IV, 2215B Garland Ave., Nashville, TN 37232-0252, Phone 615-343-5675, Fax 615-343-6229, keith.wilson@vanderbilt.edu.

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Author contributions:

Rupesh Chaturvedi: study concept and design, acquisition of data, analysis and interpretation of data, drafting of the manuscript, statistical analysis

Mohammad Asim: acquisition of data; technical support

Judith Romero-Gallo: material support, acquisition of data

Daniel P. Barry: critical revision of manuscript for intellectual content

Svea Hoge: acquisition of data

Thibaut de Sablet: acquisition of data

Alberto G. Delgado: acquisition of data

Lydia E. Wroblewski: acquisition of data

M. Blanca Piazuolo: acquisition of data

Fang Yan: material support, acquisition of data

Dawn A. Israel: material support, critical revision of manuscript for intellectual content

Robert A. Casero, Jr.: material support, and critical revision of manuscript for intellectual content

Pelayo Correa: material support, obtained funding

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Keith T. Wilson: study concept and design, analysis and interpretation of data, drafting of the manuscript, critical revision of manuscript for important intellectual content, statistical analysis, obtained funding, study supervision

Abstract

BACKGROUND & AIMS—*Helicobacter pylori*-induced gastric carcinogenesis has been linked to the microbial oncoprotein CagA. Spermine oxidase (SMO) metabolizes the polyamine spermine into spermidine and generates H₂O₂ that causes apoptosis and DNA damage. We determined if pathogenic effects of CagA are attributable to SMO.

METHODS—Levels of SMO, apoptosis, and DNA damage (8-oxoguanosine) were measured in gastric epithelial cell lines infected with *cagA*⁺ or *cagA*[−] *H. pylori* strains, or transfected with a CagA expression plasmid, in the absence or presence of SMO small interfering RNA, or an SMO inhibitor. The role of CagA in induction of SMO and DNA damage was assessed in *H. pylori*-infected gastritis tissues from humans, gerbils, and both wild-type and hypergastrinemic INS-GAS mice, using immunohistochemistry and flow cytometry.

RESULTS—*cagA*⁺ strains or ectopic expression of CagA, but not *cagA*[−] strains, led to increased levels of SMO, apoptosis, and DNA damage in gastric epithelial cells, and knockdown or inhibition of SMO blocked apoptosis and DNA damage. There was increased SMO expression, apoptosis, and DNA damage in gastric tissues from humans infected with *cagA*⁺, but not *cagA*[−] strains. In gerbils and mice, DNA damage was CagA-dependent and present in cells that expressed SMO. Gastric epithelial cells with DNA damage that were negative for markers of apoptosis accounted for 42–69% of cells in gerbils and INS-GAS mice with dysplasia and carcinoma.

CONCLUSIONS—By inducing SMO, *H. pylori* CagA generates cells with oxidative DNA damage, and a subpopulation of these cells are resistant to apoptosis and thus at high risk for malignant transformation.

Keywords

polyamines; epithelial cells; DNA damage

Introduction

Infection with *Helicobacter pylori* is associated with an increased risk for gastric cancer¹, the second leading cause of cancer death worldwide.² *H. pylori* selectively colonizes the human gastric mucosa and infects half of the world's population, including 30–40% in the United States.³ *H. pylori* interacts with gastric epithelial cells and cause activation of signaling cascades that result in dysregulation of diverse biological functions.^{3,4} Infection with *H. pylori* causes an increase in gastric epithelial apoptosis in vitro and in tissues from infected patients,^{5,6} and this has been implicated in gastric carcinogenesis.^{3,4} However, *H. pylori* infection increases expression of pro- and anti-apoptotic proteins,⁶ and epidermal growth factor receptor activation by *H. pylori* limits the amount of gastric epithelial cell apoptosis in cell lines and infected mice.⁷ We have demonstrated that polyamines mediate *H. pylori*-induced apoptosis, and this occurs by upregulation of spermine oxidase (SMO), which back-converts the polyamine spermine to spermidine, and produces H₂O₂ in this process that causes mitochondrial membrane depolarization and caspase activation leading to apoptosis.^{8,9}

Oxidative stress that occurs during interactions between *H. pylori* and gastric epithelial cells can lead to DNA damage in vivo in humans and mice by causing nucleotide oxidation.^{10,11} Chronic *H. pylori* infection increases mutation frequency leading to base substitutions and frame shift mutations¹² and accumulation of cells with damaged DNA may occur by downregulation of pro-apoptotic proteins.¹³ These findings highlight the importance of oxidative stress during *H. pylori* infection, as the fate of cells with damaged DNA is critical in the development of *H. pylori*-induced cancer.

The *cag* pathogenicity island of *H. pylori* has been implicated in disruption of gastric epithelial cell function and morphology.^{3, 4, 14} The *cag* island comprises 31 putative genes, including *cagA*, encoding an effector molecule (CagA), and others, including *cagE*, that are required for assembly of a type IV secretion system that delivers CagA into gastric epithelial cells.^{3, 4, 15} Subjects infected with *cagA*⁺ *H. pylori* strains exhibit higher levels of gastric mucosal inflammation, and increased risk of development of premalignant atrophic gastritis and intestinal metaplasia.¹⁶ CagA seropositivity is associated with an increased risk of gastric cancer compared to *cagA*⁻ infection,^{17, 18} and advanced tumor stage.^{16, 18} Moreover, CagA overexpressing transgenic mice develop gastric adenocarcinomas.¹⁹ The biochemical and cellular mechanisms responsible for the association between CagA and gastric carcinogenesis have not been defined. We now demonstrate that *H. pylori* CagA induces DNA damage and apoptosis in gastric epithelial cells in vitro and in vivo in a SMO-dependent manner. In addition, in the gerbil and Insulin-Gastrin (INS-GAS) models of gastric cancer, a substantial percentage of SMO-expressing cells with DNA damage are resistant to apoptosis. Thus, we have identified a molecular mechanism for CagA-associated gastric carcinogenesis.

Materials and Methods

Reagents

See Supplementary Methods.

Bacteria

The human *H. pylori* gastric ulcer strain B128,²⁰ its gerbil-adapted oncogenic derivative 7.13,²⁰ isogenic mutants of 7.13,²⁰ as well as strain 60190 and its *cagA* isogenic mutant²¹ were used. Strain PMSS1 and its *cagE* isogenic mutant was provided by M. Amieva.²²

Cell and Culture Conditions

Mouse conditionally-immortalized stomach epithelial (ImSt) cells^{7, 23} and human gastric cancer AGS cells were grown and co-cultured with *H. pylori*.^{7, 9}

Animal Infections

C57BL/6 mice were infected with wild-type and *cagE*⁻ PMSS1,²² and gerbils were infected with 7.13 or its isogenic *cagA* mutant.²⁴ Insulin-Gastrin (INS-GAS) mice were infected with PMSS1.

Human Subjects

Gastric tissues were used as described,²⁰ and as detailed in Supplementary Methods.

Additional Molecular Assays

See Supplementary Methods.

Transient Transfection of *cagA*

The plasmid pSP65SR α containing *cagA* was transfected in ImSt cells; see Supplementary Methods.

Immunohistochemistry for SMO and 8-hydroxy-2'-deoxyguanosine (8-OHdG)

See Supplementary Methods.

Detection of Apoptosis in Gastric Tissues

Apoptotic cells were identified by *in situ* oligo ligation (ISOL).⁷

Isolation of Epithelial Cells from Gastric Tissue

Gastric epithelial cells were isolated from frozen samples by dissociation and dispersion.²³

Statistical Analysis

Quantitative data are shown as the mean $\pm$ SE. Analyses are in the Supplementary Methods.

Results

Spermine Oxidase Induction by *H. pylori* Is CagA-Dependent

We have previously implicated SMO induction by *H. pylori* in the oxidative stress that occurs in infected gastric cancer epithelial cell lines.⁹ In order to address the role of CagA in SMO induction and its biological effects, we now used conditionally-immortalized gastric epithelial cells (ImSt cells) exposed to the carcinogenic *H. pylori* strain 7.13. When ImSt cells were co-cultured with 7.13, higher levels of CagA were detected, compared to when its non-carcinogenic parental strain B128 was tested (Supplementary Figure 1A).²⁰ Co-culture of 7.13 with ImSt cells increased SMO mRNA expression by 8-fold, whereas B128 caused less than a 3-fold increase, and a *cagA* isogenic mutant of 7.13 (7.13 *cagA*⁻) did not induce SMO mRNA expression (Figure 1A). Similarly, when assessed by Western blotting, 7.13 significantly upregulated SMO protein levels, which were induced to a lesser degree with B128, and 7.13 *cagA*⁻ did not increase SMO protein levels (Figure 1B). We confirmed these findings with flow cytometric analysis of SMO protein levels (Figure 1C) and with immunofluorescence staining for SMO (Figure 1D). ImSt cells co-cultured with 7.13 displayed intense cytoplasmic accumulation of SMO protein with perinuclear localization also apparent (Figure 1D); SMO staining was substantially less in cells cultured with B128 and absent when 7.13 *cagA*⁻ was used. 7.13 *cagE*⁻ also failed to induce SMO expression, whereas mutants of *flaA*, *ureA*, or *slt*, which inhibits peptidoglycan synthesis, induced SMO mRNA expression to the same level as wild-type 7.13 (Figure 1E).

In addition, 7.13 induced SMO mRNA expression in a manner that was dependent on the multiplicity of infection (MOI) in ImSt cells, and 7.13 *cagA*⁻ failed to induce SMO expression at each MOI from 10–200 (Supplementary Figure 1B). To test strain and cell line effects, we also co-cultured human AGS cells with *H. pylori* 60190, a *cagA*⁺ laboratory-adapted clinical strain²¹, and found that induction of SMO mRNA expression was significantly attenuated with the 60190 *cagA*⁻ isogenic mutant in these cells, similar to the results with strain 7.13 (Supplementary Figure 1C).

H. pylori CagA-Induced Apoptosis Is SMO-Dependent

Our previous studies indicated that *H. pylori*-induced SMO expression and activity correlates with the time course of apoptosis in transformed AGS gastric epithelial cells.⁹ We assessed the role of SMO and *cagA* in ImSt cells, which exhibit a normal phenotype at 37°C.²⁸ Co-culture of 7.13 with ImSt cells resulted in a significant increase in H₂O₂ production, whereas B128 and 7.13 *cagA*⁻ did not cause this effect (Figure 2A). Consistent with our previous report that H₂O₂ derived from SMO results in apoptosis,^{8,9} 7.13 induced significantly more apoptosis than B128 or 7.13 *cagA*⁻ (Figure 2B and C). The level of apoptosis induced by strain 7.13 increased in an MOI-dependent manner, and 7.13 *cagA*⁻ induced significantly less apoptosis throughout the MOI range tested from 10–200 (Supplementary Figure 2A). When assessed in AGS cells, strain 60190-induced apoptosis was also significantly attenuated when its *cagA*⁻ isogenic mutant was used (Supplementary

Figure 2B). When ImSt cells were transfected with SMO siRNA (knockdown depicted in Supplementary Figure 3A and B), H₂O₂ and apoptosis were effectively reduced in cells stimulated with 7.13, while there was no reduction in cells stimulated with 7.13 *cagA*⁻, and only a small decrease in cells co-cultured with B128 (Figure 2D and E; Supplementary Figure 3C). Taken together, these data indicate that the CagA-dependent component of the apoptosis is mediated by SMO.

H. pylori-Induced DNA Damage Is Mediated by SMO and Is CagA-Dependent

We utilized a specific assay for oxidative DNA damage, in which a FITC-labeled peptide that binds to 8-oxoguanosine is detected by flow cytometry.⁹ Strain 7.13 increased DNA damage, while B128 induced lower levels, and 7.13 *cagA*⁻ did not cause DNA damage (Figure 3A and B). Strain 7.13 increased levels of DNA damage in a MOI-dependent manner that plateaued at MOI 200, and 7.13 *cagA*⁻ failed to increase DNA damage at each MOI (Supplementary Figure 4A). When AGS cells were co-cultured with *H. pylori* 60190, there was also increased DNA damage, while 60190 *cagA*⁻ had no effect (Supplementary Figure 4B). Knockdown of SMO by siRNA in ImSt cells resulted in a marked reduction in 7.13-induced DNA damage, whereas this knockdown resulted in only small decreases in DNA damage in cells co-cultured with the 7.13 *cagA*⁻ or B128 strains (Figure 3C and D).

Ectopic Expression of CagA Increases SMO Expression, Apoptosis, and DNA Damage

We directly tested the effects of CagA in ImSt cells, using co-transfection of *cagA* and EGFP expression vectors, and analyzing cells expressing EGFP, indicative of successful transfection, by flow cytometry. Exogenous expression of *cagA* increased levels of CagA protein (Figure 4A), SMO protein (Figure 4B) and apoptosis (Figure 4C and D) in parallel, when compared to empty vector alone. MDL 72527, an inhibitor of SMO,⁹ decreased CagA-induced apoptosis (Figure 4C and D). Similarly, expression of CagA increased DNA damage that was inhibited with MDL 72527 (Figure 4E and F). EGFP-negative cells did not show increases in levels of SMO protein, apoptosis, or DNA damage (Supplementary Figure 5A–C).

H. pylori Infection Increases SMO Expression, DNA Damage, and Apoptosis in Human Gastric Tissues in a CagA-Dependent Manner

To provide further biological relevance, we determined levels of SMO, DNA damage, and apoptosis in gastric biopsies from human subjects in which *H. pylori* infection status and the presence or absence of *cagA* in cultured isolates was assessed by PCR.²⁰ Two of the eight strains that were PCR-positive did not express CagA protein (Supplementary Figure 6A); these strains were classified as *cagA*⁻. Using a TaqMan-based assay²⁵ there was a substantial (two-log-order) increase in SMO mRNA expression levels in gastric biopsies from patients infected with *cagA*⁺ *H. pylori* compared to normal gastric tissues, and these levels were not upregulated in tissues from patients infected with *cagA*⁻ strains (Figure 5A). When co-cultured with AGS cells, all clinical isolates expressing CagA translocated this protein, as indicated by detectable phosphorylated CagA in cell lysates (Supplementary Figure 6B). To assess functionality of CagA in vivo, IL-8 mRNA expression in the human gastric tissues was measured (Supplementary Figure 6C) and it paralleled the levels of CagA and phosphorylated CagA in AGS cells. There was increased SMO mRNA expression in biopsies from patients infected with *cagA*⁺ *H. pylori* strains (Figure 5A). SMO mRNA levels in gastric tissues were positively correlated with the amounts of CagA or phosphorylated CagA detected in AGS cell lysates co-cultured with these strains (Supplementary Figure 6D and E).

Immunostaining of gastric tissues indicated increased SMO protein levels in epithelial cells from patients infected with *cagA*⁺ *H. pylori* compared to normal subjects or those infected

with *cagA*⁻ strains (Figure 5A–C). As shown in Figure 5B, SMO staining in normal gastric tissues was weak and restricted to the surface epithelium, was strongly increased in surface epithelial cells and within deep glands in patients infected with *cagA*⁺ *H. pylori*, and was diminished in patients infected with *cagA*⁻ *H. pylori*. Both cytoplasmic and nuclear localization of SMO was detected on high power images (Figure 5C). There was also strong staining for the DNA damage marker, 8-OHdG (Figure 5D; data quantified in Supplementary Figure 7), and for apoptosis, assessed by the ISOL technique (Figure 5E), in gastric biopsies from patients infected with *cagA*⁺ *H. pylori* that was markedly decreased in those infected with *cagA*⁻ *H. pylori* or were uninfected.

SMO Expression and DNA Damage Correlate in Epithelial Cells Isolated from Human Gastric Biopsies

To further establish the relationship between SMO expression and DNA damage, we isolated epithelial cells from human gastric biopsies. When examined by flow cytometry, cells from patients infected with *cagA*⁺ *H. pylori* strains exhibited increased SMO protein expression and oxidative DNA damage (8-oxoguanosine levels) compared to cells isolated from normal subjects or patients harboring *cagA*⁻ strains (Figure 5F). A plot of SMO versus 8-oxoguanosine levels demonstrated a significant positive correlation (Figure 5F).

DNA Damage in Gerbils Is CagA-dependent and Is Present in a Subpopulation of Cells That Are Resistant to Apoptosis

Gerbils infected with *H. pylori* 7.13 developed gastric dysplasia or carcinoma (Supplementary Figure 8). When gastric epithelial cells isolated from these animals were analyzed by flow cytometry, there were increased SMO protein and 8-oxoguanosine levels that were strongly correlated, while cells from animals infected with the 7.13 *cagA*⁻ strain and possessing only gastritis exhibited no such increases (Figure 6A, 6B; Supplementary Figure 8). As shown in Figure 6B, the majority of the 8-oxoguanosine^{high} cells from 7.13-infected gerbils were SMO^{high} ($93.8 \pm 0.9\%$; representative plots in Supplementary Figure 9). Neoplastic transformation of cells requires DNA alterations as well as circumvention of apoptosis. To monitor such changes we simultaneously analyzed active caspase-3, a marker of apoptosis, and DNA damage in the same cells. Wild-type 7.13, but not 7.13 *cagA*⁻, caused an increase in both markers, including a subset of cells that were 8-oxoguanosine^{high}, active caspase-3^{high} (Figure 6C and 6D). Importantly, a substantial percentage of the 8-oxoguanosine^{high} cells were also active caspase-3^{low} ($44.2 \pm 3.1\%$; representative plots in Supplementary Figure 10), identifying a subpopulation of cells with DNA damage that were apoptosis-resistant.

H. pylori Induces SMO in Mice in a CagA-dependent Manner and Also Leads to Generation of Cells With DNA Damage and Resistance to Apoptosis

H. pylori strain PMSS1 can translocate CagA into gastric epithelial cells after in vivo passage and is an excellent colonizer of mice.²² We examined gastric epithelial cells isolated from mice 2 months after infection of 6–8 week old mice with PMSS1 or its *cagE* isogenic mutant that fails to translocate CagA,²² and found that PMSS1 infection led to increased levels of SMO protein, active caspase-3, and 8-oxoguanosine, whereas cells from mice infected with PMSS1 *cagE*⁻ exhibited no significant increase in these parameters (Figure 7A; Supplementary Figure 11A–C). There were no differences in colonization levels between wild-type and *cagE*⁻ PMSS1 (data not shown). There was a significant positive correlation between SMO and 8-oxoguanosine levels (Figure 7A) and the increase in the latter with PMSS1 infection was detected in cells expressing high levels of SMO and was absent in mice infected with PMSS1 *cagE*⁻ (Supplementary Figure 11B).

We used hypergastrinemic INS-GAS mice to assess levels of SMO and DNA damage in gastric epithelial cells because these mice develop neoplasia (Figure 7B). In the uninfected mice with dysplasia, the percentage of cells that were SMO^{high}, 8-oxoguanosine^{high} was low compared to the percentage in the *H. pylori* PMSS1-infected INS-GAS mice (Figure 7C). Most of the increase in the percentage of cells with DNA damage with infection derived from the SMO^{high} population (Figure 7C; Supplementary Figure 12). The SMO^{high}, 8-oxoguanosine^{high} cell population was further increased with carcinoma (Figure 7C). A substantial population of 8-oxoguanosine^{high}, active-caspase-3^{low} cells was present in PMSS1-infected INS-GAS mice with carcinoma; this population was more modest in infected mice with dysplasia and nearly absent ($2.6 \pm 1.7\%$ of cells) in uninfected mice (Figure 7D).

Additionally, incubation of ImSt cells with 7.13 increased the population of 8-oxoguanosine^{high}, active-caspase-3^{low} cells ($19.8 \pm 2.3\%$ of cells) in vitro, while 7.13 *cagA*⁻ did not increase these cells above basal levels (Supplementary Figure 13A). The 8-oxoguanosine^{high}, active-caspase-3^{low} cells expressed higher levels of the anti-apoptotic protein Bcl-2 than 8-oxoguanosine^{low}, active-caspase-3^{low} cells (Supplementary Figure 13B and 13C). Gating on active caspase-3^{low} cells (Supplementary Figure 14A) demonstrated that with 7.13 stimulation there was a significant increase in the Bcl-2^{high} population, which was not observed in cells cultured with 7.13 *cagA*⁻ (Supplementary Figure 14B–C). Further, 8-oxoguanosine levels were increased in the active caspase-3^{low}, Bcl-2^{high} versus active caspase-3^{low}, Bcl-2^{low} cells (Supplementary Figure 14D).

Discussion

Our current findings implicate SMO as a causal link between CagA and gastric carcinogenesis. We have shown that in non-transformed gastric epithelial cells, *H. pylori* strain 7.13 induces more SMO expression and associated H₂O₂ production, apoptosis, and DNA damage than the parental strain B128, which is a poor translocator of CagA. The essential role of CagA was supported by our findings that *cagA* isogenic mutants of strains 7.13 and 60190 exhibited profound attenuation of SMO inducing ability. The role of SMO as a causal factor in generation of oxidative stress and its consequences was demonstrated by the potent effects of SMO knockdown in abrogating CagA-dependent H₂O₂ production, apoptosis, and DNA damage. The link between CagA, SMO, and its biologic effects was further evidenced by our findings that ectopic expression of *cagA* directly induced SMO, and that the associated apoptosis and DNA damage was blocked by an SMO inhibitor.

While *flaA*, *ureA*, and *slt* isogenic mutants exhibited no attenuation of SMO induction, the 7.13 *cagE*⁻ mutant resulted in reduced SMO levels similar to those occurring with the *cagA*⁻ mutant. CagE is an essential component of the *H. pylori* type IV secretion system and is required for translocation of CagA.²² *cagA*⁺ *H. pylori* strains are associated with increased production of reactive oxygen species (ROS) in human gastric mucosa,²⁶ though no mechanism linking the induction of ROS and *cagA*⁺ infection has been elucidated. While previous studies have demonstrated increased ROS in transformed gastric epithelial cell lines,^{9, 10} we now report that H₂O₂, specifically, is increased in non-malignant ImSt cells and that this is a CagA-dependent process. Furthermore, in addition to cytoplasmic staining, SMO also localized to the perinuclear region and the nucleus of 7.13-infected ImSt cells; this finding is consistent with the report of a nuclear isoform of SMO,^{25, 27} and indicates that such proximity to cellular chromatin could increase the likelihood of DNA damage resulting from the release of H₂O₂ during spermine oxidation.

H. pylori infection has been reported to result in either increased or decreased levels of apoptosis.^{28,29} Our results demonstrate that *H. pylori*-induced apoptosis in ImSt cells is

largely CagA-dependent, since 7.13 induced high levels of apoptosis, while B128 and 7.13 *cagA*⁻ caused significantly less. However, levels were still higher with these two strains than in uninfected cells, suggesting that some apoptotic cell death is due to other bacterial factors that have been implicated as inducers in gastric epithelial cells.³⁰ Our results implicating CagA as an important inducer of apoptosis differ from a recent report that exogenous overexpression of CagA can have a protective effect on apoptosis of gastric epithelial cells induced by etoposide or VacA.³¹ One factor is that the ImSt cells we used readily exhibit apoptosis, whereas transformed cell lines such as AGS cells are relatively apoptosis-resistant. MOI may also be a factor; we reported that a low MOI of *H. pylori* protects B lymphocytes from spontaneous apoptosis, while a higher MOI has the opposite effect.³² However, in the current study we found a reduction in both apoptosis and DNA damage with *cagA*⁻ strains across a broad range of MOIs. It has been reported that ectopic expression of CagA causes apoptosis in gastric epithelial cells;³³ we now show that this occurs in a SMO-dependent manner.

In addition to dysregulation of apoptosis, another essential step in neoplastic transformation is accumulation of genetic alterations that can result from oxidative DNA damage. Studies in Big Blue transgenic mice demonstrated that *H. pylori* infection causes base substitution and frame-shift mutations.¹² While *cagA*⁺ infection has been linked to DNA damage in humans,³⁴ the responsible biochemical and cellular mechanisms have not been identified. We have directly implicated SMO in this process in epithelial cells. This was shown by siRNA knockdown and inhibitor studies in vitro, and our findings that exogenous overexpression of CagA caused epithelial cell DNA damage that could be prevented with an SMO inhibitor.

We demonstrated the in vivo relevance of our findings as we observed increased SMO mRNA expression and intense nuclear and perinuclear SMO staining in biopsies from humans infected with *cagA*⁺, but not *cagA*⁻ strains. By both immunostaining and flow cytometry, we demonstrated more oxidative DNA damage in gastric epithelial cells with high levels of SMO expression; the strong correlation between these two events indicates that they are linked. Neutrophils and macrophages produce ROS during the *H. pylori* immune response⁸ that may also damage DNA of adjacent gastric epithelial cells. However, ROS produced within gastric epithelial cells by SMO would be expected to be more damaging because of their close proximity to the DNA, and they may thus be an important factor in tumorigenesis. Also, the increase in epithelial SMO we show in vivo may be a specific response to *H. pylori*, as we have reported that in colon tissues from patients with ulcerative colitis, SMO was primarily upregulated in mononuclear cells, but not epithelial cells.²⁵ We also demonstrated a strong link between CagA, SMO, and DNA damage in two experimental models of *H. pylori* infection, namely mice infected with *H. pylori* PMSS1, which translocates CagA,²² and gerbils infected with 7.13. In both models the wild-type strains resulted in a strong correlation between SMO and 8-oxoguanosine levels, and mice infected with PMSS1 *cagE*⁻, a strain incapable of CagA translocation,²² and gerbils infected with 7.13 *cagA*⁻ exhibited markedly lower levels of SMO and oxidative DNA damage, providing further evidence that CagA plays a major role in the induction of SMO-driven DNA damage.

Dysregulation of apoptosis has the potential to be deleterious, since increased apoptosis would be expected to increase the likelihood of development of gastric atrophy and potentially, increased epithelial cell turnover, while protection from apoptosis could increase risk for neoplastic transformation.⁴ Varying results have been reported related to the effect of CagA on apoptosis in the human stomach^{28,29}, some of which found no association between CagA and increased apoptosis during infection. In the current report, we found a modest increase in apoptosis in our human samples with gastritis. Such differing results may

be due to differences in the methodology used to detect apoptosis, location of biopsies, and histopathologic stage of disease. In the gerbil model of gastric dysplasia and carcinoma, we discovered that while infection with the *cagA*⁺ strain 7.13 also resulted in more epithelial apoptosis than *cagA*⁻ infection, there was a subpopulation of cells that exhibited high levels of DNA damage and low levels of apoptosis (8-oxoguanosine^{high}, active-caspase-3^{low}) that was only present in cells from gerbils infected with the *cagA*⁺ strain. Moreover, we also observed this subpopulation of cells in INS-GAS mice infected with PMSS1, another strain capable of translocating CagA into gastric epithelial cells, as well as in ImSt cells infected with 7.13, but not with 7.13 *cagA*⁻. Taken together, these findings indicate that while *H. pylori* causes apoptosis in gastric epithelial cells, there is also a substantial fraction of cells infected with *cagA*⁺ strains that are protected from apoptosis during infection.

These differences in susceptibility to apoptosis may derive in part from the heterogeneity of the gastric epithelial cell populations in the stomach,³⁵ but another important factor may be alterations in pro- and anti-apoptotic signaling pathways.⁴ In the present study, we found that 8-oxoguanosine^{high}, active caspase-3^{low} populations of ImSt cells activated with 7.13 had increased expression of anti-apoptotic Bcl-2. Our group has reported that epidermal growth factor receptor activation during *H. pylori* infection protects gastric epithelial cells from apoptosis.⁷ Moreover, we have found that inhibition of EGFR transactivation attenuates both SMO expression and the 8-oxoguanosine^{high}, active caspase-3^{low} cell population that we have described herein (data not shown).

Gastric carcinogenesis is a multifactorial disease and other contributors to the pathologic process could also be involved, including include epigenetic changes occurring within the epithelium,³⁶ changes in specific genes in the gastric microenvironment,³⁷ or expansion of stomach pluripotent stem cells³⁸ or bone marrow-derived stem cells.³⁹ Thus, a compensatory increase in proliferation of stem cells in the stomach due to increased epithelial cell apoptosis during *H. pylori* infection may contribute to neoplastic potential. The current study provides insights into the mechanisms of interactions between *H. pylori* CagA and the biochemical pathways of DNA damage in epithelial cells. These findings may be useful in identifying new therapies to prevent gastric carcinogenesis, including interference with polyamine synthesis⁴⁰ to prevent generation of SMO substrate, or direct inhibition of SMO.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

SMO	spermine oxidase
CagA	cytotoxin-associated gene A
PMSS1	pre-mouse Sydney strain 1

INS-GAS	Insulin-Gastrin
EGFP	enhanced green fluorescence protein
ISOL	<i>in situ</i> oligo ligation
PI	propidium iodide
8-OHdG	8-hydroxy-2'-deoxyguanosine
ImSt	immortalized stomach
FITC	fluorescein isothiocyanate
MFU	mean fluorescence units
MOI	multiplicity of infection
PCR	polymerase chain reaction
Scr	scrambled
siRNA	small interfering RNA
ROS	reactive oxygen species

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Figure 1.

Induction of SMO in ImSt cells co-cultured with *H. pylori* strains at a MOI of 200. (A) Real-time PCR (upper panel), and semi-quantitative reverse transcription PCR (lower panel) for SMO mRNA expression assessed at 6 h. (B) Upper panel, densitometry; lower panel, representative Western blot for SMO and β-actin. (C) Flow cytometric analysis of SMO protein. (D) Immunofluorescent detection of SMO; cells were stained for SMO and β-actin, detected with secondary antibodies conjugated with FITC (green) and rhodamine (red), respectively. Nuclei were stained with DAPI (blue). Examples of perinuclear and nuclear staining for SMO are denoted by white arrows. (E) Cells were cultured with isogenic mutants of 7.13. SMO mRNA expression was measured by real-time PCR after 6 h. For A,

B, and *E*, $n = 3$ in duplicate; *** $P < .001$ versus control (Ctrl); ## $P < .01$ versus B128; §§ $P < .01$, §§§ $P < .001$ versus 7.13.

Figure 2.

Induction of H₂O₂ and apoptosis in ImSt cells by *H. pylori* and effect of SMO siRNA. Cells were co-cultured with *H. pylori* at an MOI of 200 for 24 h. (A) H₂O₂ in supernatants. (B) Summary data for apoptosis, and (C) Representative dot plots; percent of cells in the annexin V⁺/propidium iodide (PI)⁺ (late apoptosis) quadrants are indicated. (D) Levels of H₂O₂ in supernatants and (E) summary apoptosis data in cells transfected with scrambled (Scr) siRNA or SMO siRNA. In A, B, D, and E, n = 3 in duplicate. For A and B, ***P* < .01, ****P* < .001 versus control; ##*P* < .01, ###*P* < .001 versus B128; and §§*P* < .01, §§§*P* < .001 versus 7.13. For D and E, **P* < .05, ***P* < .01 versus uninfected cells transfected with

scrambled siRNA; § $P < .05$, §§ $P < .01$ versus cells transfected with scrambled siRNA and incubated with 7.13.

Figure 3.

Induction of oxidative DNA damage in ImSt cells by *H. pylori* and effect of SMO siRNA. Cells were co-cultured for 24 h with *H. pylori* at MOI 200. (A) Summary data for 8-oxoguanosine levels, in mean fluorescence units (MFU), measured by flow cytometry. (B) Representative histogram for 8-oxoguanosine levels. (C) Summary data for DNA damage in cells transfected with siRNA. (D) Representative histograms for 8-oxoguanosine levels in transfected cells. In A and C, n = 3 in duplicate. For A, * $P < .05$ versus control, # $P < .05$ versus B128, § $P < .05$ versus 7.13. For C, * $P < .05$, *** $P < .001$ versus uninfected ImSt cells transfected with scrambled siRNA; § $P < .05$, §§ $P < .01$ versus cells transfected with scrambled siRNA and incubated with 7.13.

Figure 4.

Ectopic expression of *cagA* in ImSt cells and effect on apoptosis and DNA damage. (A) Representative Western blot for CagA and β -actin in ImSt cells transfected with empty vector and CagA expression vector. (B–F) Cells were co-transfected with EGFP and *cagA* vectors, and data were analyzed from EGFP⁺ cells 24 h later. (B) SMO expression levels analyzed by flow cytometry. (C) Summary apoptosis data in cells $\pm$ the SMO inhibitor MDL 72527 (250 μ M). (D) Representative dot plots of annexin V versus PI for apoptosis measurement. (E) Summary data for oxidative DNA damage. (F) Representative histogram for 8-oxoguanosine levels. In B, C, and E, n = 2 in duplicate; ** P < .01, *** P < .001 versus

cells transfected with empty vector; in *C* and *E*, §§ $P < .01$ versus cells transfected with *cagA* vector.

Figure 5.

Levels of SMO, DNA damage, and apoptosis in human antral gastric biopsies. (A) *Left panel*: SMO mRNA expression measured by TaqMan PCR; red diamonds indicate strains PCR-positive for *cagA*, but CagA protein-negative (Supplementary Figure 6A); *right panel*: scoring of SMO staining intensity in epithelial cells from immunohistochemistry analysis. (B, and C) Immunohistochemistry for SMO. (D) Immunohistochemistry for 8-OHdG. (E) Levels of apoptosis by ISOL, imaged by DIC microscopy. (F) Gastric epithelial cells were isolated from gastric tissues and analyzed by flow cytometry. *Left panel*: levels of SMO; *middle panel*: levels of DNA damage; *right panel*: correlation (Pearson coefficient) between SMO and DNA damage levels. In A and F, each circle is a different patient; ** $P < .01$, *** $P < .001$ versus uninfected normal tissues, § $P < .05$, §§ $P < .01$, §§§ $P < 0.001$ versus *cagA*-positive tissues.

Figure 6.

Levels of SMO protein, DNA damage, and apoptosis in gerbils infected with 7.13 or 7.13 *cagA*⁻ at 14 weeks post-inoculation. In the 7.13 group, 5 gerbils had invasive carcinoma and 5 had dysplasia, while in the 7.13 *cagA*⁻ group all had chronic gastritis only. (A) *Left and middle panels*: levels of SMO protein and 8-oxoguanosine, respectively, measured by flow cytometry; *right panel*: correlation (Pearson coefficient) between SMO and 8-oxoguanosine levels. (B) Representative dot plots. (C) Percentage of cells active caspase-3^{high} (*left panel*); 8-oxoguanosine^{high} (*middle panel*); active caspase-3^{low}, 8-oxoguanosine^{high} (*right panel*). (D) Representative dot plots. In A and C, each circle is a different gerbil; ** $P < .01$, *** $P < .001$ versus uninfected control; §§ $P < .01$, §§§ $P < .001$ versus gerbils infected with 7.13.

Figure 7.

SMO and DNA damage levels in gastric epithelial cells in mice. (A) C57BL/6 mice were infected with PMSS1 or its *cagE* isogenic mutant, and gastric epithelial cells were isolated at 2 months post-inoculation. *Left and middle panels*: summary data for levels of SMO protein and 8-oxoguanosine, respectively, measured by flow cytometry. *Right panel*: correlation (Spearman coefficient) between levels of SMO protein and 8-oxoguanosine. (B–D) FVB/N INS-GAS mice infected with PMSS1 for 4 months. (B) H&E staining showing dysplastic and dilated glands in infected and uninfected mice, and invasive carcinoma in *H. pylori*-infected mice. (C) Representative dot plots of SMO and 8-oxoguanosine levels in gastric epithelial cells. (D) Representative dot plots of active caspase-3 and 8-oxoguanosine levels.

In *A*, $n = 5$ per group; In *B–D*, $n = 3$ uninfected and 6 infected mice. For *A*, $**P < .01$ versus control, $§§P < .01$ versus PMSS1.