Spermine Oxidation Induced by *Helicobacter pylori* Results in Apoptosis and DNA Damage: Implications for Gastric Carcinogenesis

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Abstract

Oxidative stress is linked to carcinogenesis due to its ability to damage DNA. The human gastric pathogen *Helicobacter pylori* exerts much of its pathogenicity by inducing apoptosis and DNA damage in host gastric epithelial cells. Polyamines are abundant in epithelial cells, and when oxidized by the inducible spermine oxidase SMO(PAOh1) H₂O₂ is generated. Here, we report that *H. pylori* up-regulates mRNA expression, promoter activity, and enzyme activity of SMO(PAOh1) in human gastric epithelial cells, resulting in DNA damage and apoptosis. *H. pylori*-induced H₂O₂ generation and apoptosis in these cells was equally attenuated by an inhibitor of SMO(PAOh1), by catalase, and by transient transfection with small interfering RNA targeting SMO(PAOh1). Conversely, SMO(PAOh1) overexpression induced apoptosis to the same levels as caused by *H. pylori*. Importantly, in *H. pylori*-infected tissues, there was increased expression of SMO(PAOh1) in both human and mouse gastritis. Laser capture microdissection of human gastric epithelial cells demonstrated expression of SMO(PAOh1) that was significantly attenuated by *H. pylori* eradication. These results identify a pathway for oxidative stress-induced epithelial cell apoptosis and DNA damage due to SMO(PAOh1) activation by *H. pylori* that may contribute to the pathogenesis of the infection and development of gastric cancer.

Introduction

*Helicobacter pylori* is a Gram-negative microaerophilic bacterium that selectively colonizes the human stomach and causes chronic gastritis, peptic ulcers, and gastric cancer. Despite inciting substantial acute and chronic immune and inflammatory responses, *H. pylori* infection generally persists for the life of the host, in part due to its ability to evade the antimicrobial effects of the immune response (1).

We have recently reported that one potential cause of the ineffective immune response is the induction of apoptosis in macrophages caused by oxidation of polyamines resulting in generation of H₂O₂ (2). Apoptosis of gastric epithelial cells in *H. pylori* infection has been an area of focus in both *in vivo* (3) and *in vitro* studies (4). It may be an important contributing factor in the increased epithelial permeability and mucosal damage, and it has been associated with compensatory cell proliferation (5), all of which contribute to both the inflammation and risk for carcinogenesis. Additionally, DNA damage has been reported in *H. pylori*-infected gastric epithelial cells in *vitro* (6) and *in vivo* (7). We now report that apoptosis and DNA damage in gastric epithelial cells infected with *H. pylori* are mediated by spermine oxidase [SMO(PAOh1); refs. 8 and 9]. SMO(PAOh1) expression and activity are induced by *H. pylori*; the resulting H₂O₂ generation, apoptosis, and DNA damage are blocked by inhibition of polyamine oxidation; and silencing of SMO(PAOh1) expression prevents apoptosis and DNA damage. Our data are the first to demonstrate the induction of polyamine oxidation by a microbial pathogen and to link oxidative stress by this pathway to apoptosis, DNA damage, and potentially carcinogenesis.

Materials and Methods

**Bacteria and Cells.** *H. pylori* strains 60190 and SS1 were grown under microaerobic conditions as described previously (10). The human gastric epithelial cell line AGS was grown in F12 medium (11). Experiments were performed in antibiotic-free medium with 10% fetal bovine serum.

**Reverse Transcription-Polymerase Chain Reaction and Real-Time Polymerase Chain Reaction.** Total RNA was isolated from AGS cells and cDNA synthesized, and primer sequences and polymerase chain reaction (PCR) product sizes for β-actin and PAO1 [mouse homologue of SMO(PAOh1)] and PCR conditions for the multiplex reactions were as reported (2). Primer sequences for human SMO(PAOh1) were: sense, 5'-GACCACAAATCAGGACACTG-3', and antisense, 5'-TATGACACCATGCAGACG-3', yielding a 160-bp product. Real-time PCR was performed using SYBR Green (2). For AGS experiments, relative expression of SMO(PAOh1) was determined using β-actin as the internal control (2). In tissue studies, SMO(PAOh1) expression was normalized to 18S rRNA.

**SMO(PAOh1) Promoter Activity.** A genomic DNA plasmid library was constructed from human AS49 adenocarcinoma cell DNA in the pBluescript SK(−) plasmid and screened with a cDNA probe homologous to exon 1 of SMO(PAOh1). From this library, a clone containing ~4479 bp to the transcriptional start site was identified. Deletion constructs were generated by restriction enzyme digestion and subcloned into pG-L2 basic. AGS cells were transiently transfected (2) with 200 ng of the above constructs and luciferase activity performed according to the manufacturer’s instructions (Promega, Madison, WI).

**SMO(PAOh1) Activity.** Lysates of AGS cells were analyzed by a chromiluminescence assay as described previously (2, 12), and the activity was expressed as nanomoles of H₂O₂ per minute per milligram protein.

**Measurement of H₂O₂.** AGS cells were incubated with 10 μmol/L CM-H₂DCFDA and intracellular H₂O₂ detected by flow cytometry as described previously (2). For measurement of H₂O₂ in supernatants, 5 × 10⁵ cells were plated in 24-well plates. After stimulation, cells were washed and incubated with 50 μmol/L Amplex Red reagent (Molecular Probes, Eugene OR) and 0.1 unit/mL horseradish peroxidase for 30 minutes at 37°C. Plates were read using a microplate reader at 560 nm, and a standard curve with varying dilutions of H₂O₂ was used (2).

**Assessment of Apoptosis.** Apoptosis was assayed using an annexin V-fluorescein isothiocyanate apoptosis detection kit (Oncogene Research Products, San Diego, CA) according to the manufacturer’s instructions.
(1 × 10^4) were analyzed by flow cytometry (2). Apoptosis was also assessed by enzyme-linked immunosorbent assay of cytoplasmic histone-associated DNA fragments (13).

**Transient Transfection of SMO(PAOh1).** AGS cells were transfected with 200 ng of pcDNA3.1-SMO(PAOh1) using LipofectAMINE PLUS and optiMEM medium (2). Transfection efficiency was determined by fluorescence in cells transfected with 400 ng of pRES2-EGFP (Clontech, Palo Alto, CA).

**Transient Transfection of SMO(PAOh1) Small Interfering RNA.** Small interfering RNA duplexes were used that targeted SMO(PAOh1) nucleotides 468 to 486, numbered from the start codon (sense, 5'-GGACGGUGGUUGAG-GAAUUC-3'; antisense, 5'-CCUGCAACCACUCCUAAAG-3'). Scrambled small interfering RNA with no sequence homology to any known genes was used as the control. Transfection conditions were as described previously (2).

**DNA Damage Assays.** DNA damage was assessed by the alkaline single-cell gel electrophoresis (comet assay) method (14). AGS cells were stimulated, trypsinized, and embedded into 0.5% low-melting agarose on glass microscope slides. After treatment with alkaline lysis buffer, slides were electrophoresed, stained with propidium iodide, and analyzed by epifluorescence microscopy. DNA damage was measured by the tail moment, defined as product of the length of the tail (in micrometers), which is DNA migrated from the nucleus, and the percentage of DNA in the tail (14).

**DNA Damage was also assessed by 8-oxoguanosine binding.** In brief, after fixation and permeabilization, cells were washed, blocked, and incubated with 8-oxoguanosine–fluorescein isothiocyanate conjugate (Kamiya Biomedical, Seattle, WA) for 1 hour in the dark. Cells were resuspended in PBS and analyzed by flow cytometry for fluorescence.

**H. pylori Gastritis Tissues.** C57BL/6 mice were infected with H. pylori SS1 and gastric tissues harvested 4 months later (13). Human gastritis samples were obtained from patients at the Baltimore Veterans Affairs Medical Center, with H. pylori status determined as described previously (15). Antral biopsies from patients with H. pylori infection from Uijongbu St. Mary Hospital (Uijongbu, Korea) were evaluated before and 2 months after confirmed H. pylori eradication. Histologic analysis after eradication demonstrated complete resolution of acute inflammation in all cases and significant reduction of chronic inflammation. Laser capture microdissection of approximately 5000 gastric epithelial cells from formalin-fixed, paraffin-embedded endoscopic gastric biopsies was performed using the Autopix automated LCM system (Arcturus, Mountain View, CA). RNA was extracted, and SMO(PAOh1) mRNA was quantified by real-time PCR and normalized for 18S rRNA.

**Statistical Analysis.** For quantitative data, values represent the mean ± SE. For comparisons between multiple groups, the Student-Newman-Keuls test was used; and for single comparisons between two groups, Student’s t test was used.

### Results

**Parallel Induction of SMO(PAOh1) and Apoptosis in H. pylori-Stimulated Human Gastric Epithelial Cells.** H. pylori infection has been linked to DNA damage and apoptosis in gastric epithelial cells (3, 4, 6, 7). However, the origin of the damaging insult has not previously been elucidated. Therefore, based on our previous results demonstrating that H. pylori induces SMO(PAOh1) in macrophages (2), we sought to determine its effects in gastric epithelial cells. H. pylori (strain 60190) induced a significant increase in SMO(PAOh1) mRNA expression in AGS cells as determined by real-time PCR analysis (Fig. 1A). Stimulation with H. pylori also resulted in a significant increase in SMO(PAOh1) promoter activity with the −1117-bp construct (Fig. 1B), indicating that the observed increase in SMO(PAOh1) mRNA is due to infection-induced transcription. There was a time-dependent increase in SMO(PAOh1) enzyme activity (Fig. 1C) that peaked at 12 hours after H. pylori stimulation. In contrast, there was no induction of activity of the acetyl PAO (ref. 16; data not shown) when assessed by a specific assay (2). To determine whether this increase in oxidase activity was accompanied by apoptosis, the sensitive technique of annexin V and propidium iodide staining of live cells was used to measure apoptosis in a highly quantitative manner.

As shown in Fig. 1C, the time course of the induction of apoptosis closely paralleled that of SMO(PAOh1) activity.

**H. pylori-Induced Spermine Oxidation Results in H2O2-Mediated Apoptosis.** To confirm that the SMO(PAOh1)-produced H2O2 was causally linked to the observed apoptosis, the effects of an oxidase inhibitor (MDL 72527), H2O2 detoxifying agent (catalase), and an SMO(PAOh1)-specific small interfering RNA were examined. H. pylori induced a significant increase in intracellular H2O2 (Fig. 1D) that was prevented by the inhibition of SMO(PAOh1) by MDL 72527 or by the addition of catalase. We also used the Amplex Red assay, specific for H2O2 in the medium, to demonstrate that H. pylori produced a significant, 2.7 ± 0.1-fold increase in extracellular H2O2 that was inhibited by 79.6 ± 7.1% with MDL 72527 and 100.4 ± 7.8% with catalase (P < 0.01 for H. pylori versus control and for H. pylori + inhibitors versus H. pylori alone; data not shown). Consistent with the H2O2 data, apoptosis induced by H. pylori was significantly attenuated by MDL 72527 and catalase, as shown in Fig. 1E and F. We also confirmed these findings by analysis of apoptosis by DNA fragmentation enzyme-linked immunosorbent assay (data not shown).

Because MDL 72527 inhibits both PAO and SMO(PAOh1) (8, 9, 16), to determine whether the spermine oxidation-mediated apoptosis was specifically due to SMO(PAOh1), we transiently transfected AGS cells with a duplex small interfering RNA specific for SMO(PAOh1). This treatment significantly inhibited H. pylori-stimulated SMO(PAOh1) mRNA expression (Fig. 2A) and produced a 79.1 ± 8.1% inhibition of SMO(PAOh1) enzyme activity (Fig. 2B). This knockdown of SMO(PAOh1) was associated with a 71.6 ± 5.8% inhibition of apoptosis (Fig. 2C).

To confirm that SMO(PAOh1) has a causal role in gastric epithelial cell apoptosis, we transiently transfected AGS cells with a full-length cDNA for SMO(PAOh1). There was a significant increase in apoptosis with SMO(PAOh1) transfection that was similar to the level of increase with H. pylori stimulation in mock-transfected cells (Fig. 2D).

**H. pylori-Induced Deoxyribonucleic Acid Damage in Gastric Epithelial Cells Is Mediated by SMO(PAOh1).** The comet assay was used to directly visualize DNA damage morphologically. There was a marked increase in the size and intensity of the tail of the DNA fluorescence of the cells in the H. pylori-treated (Fig. 3B) versus untreated cells (Fig. 3A). Treatment of AGS cells with MDL 72527 (Fig. 3C) or catalase (Fig. 3D) resulted in reduction of damage. We quantitated the tail moment in >270 cells for each condition and found that there was a 4.4 ± 0.1-fold increase with H. pylori (P < 0.01 versus control) that was inhibited by 90.3 ± 4.1% with MDL 72527 and 87.6 ± 4.2% with catalase (P < 0.01 for both inhibitors versus H. pylori alone). When we assessed 8-oxoguanosine binding by flow cytometry as an indicator of oxidatively damaged DNA, we found that stimulation with H. pylori resulted in increased fluorescence that was significantly attenuated with MDL 72527 or catalase (Fig. 3E) or transfection with SMO(PAOh1) small interfering RNA (Fig. 3F).

**SMO(PAOh1) Is Up-Regulated in H. pylori Gastritis Tissues and Down-Regulated with H. pylori Eradication.** To determine whether the in vitro observations were relevant in an in vivo setting, mouse and human tissues from H. pylori-induced gastritis were examined. There was a significant increase in mRNA expression of mouse PAO1 (Fig. 4A) and human SMO(PAOh1) (Fig. 4B) in H. pylori gastritis tissues. Levels of SMO(PAOh1) in human gastritis tissues from H. pylori-negative patients (Fig. 4B) were only modestly increased, whereas tissues from H. pylori-infected patients exhibited consistently higher levels of expression. Real-time PCR analysis in
the mouse tissues revealed a 3.8 ± 0.4-fold increase in *H. pylori* gastritis versus uninfected tissues (*P* < 0.01), and in human samples, there was a 2.5 ± 0.4-fold increase in *H. pylori*-negative gastritis and a 4.9 ± 1.5-fold increase in *H. pylori*-positive gastritis (*P* < 0.05 versus normal for *H. pylori* positive only). To confirm that SMO(PAOh1) was expressed in vivo in the gastric epithelium, we used RNA extracted from epithelial cells harvested by laser capture microdissection from *H. pylori*-infected gastric tissues (Fig. 4C). After eradication therapy, SMO(PAOh1) mRNA levels determined by real-time PCR decreased in each patient, with an 85.4 ± 7.5% inhibition compared with levels before treatment (*P* < 0.001).

**Discussion**

The involvement of oxidative stress in carcinogenesis is well established, both generally and in gastrointestinal cancers (17). However, the origins of the reactive oxygen species leading to DNA damage and cancer, in many cases, have not been identified. The results presented here describe the pathway that establishes the source of oxidative stress in human gastric epithelial cells as H$_2$O$_2$ that is specifically produced from *H. pylori*-induced spermine oxidase activity and results in both apoptosis and DNA damage. These results suggest that one link between *H. pylori* infection and gastric cancer may be SMO(PAOh1)-produced H$_2$O$_2$.

*H. pylori* infection has been reported to cause production of H$_2$O$_2$ in AGS human gastric epithelial cells, and it has been suggested that this may contribute to the carcinogenic process in *H. pylori*-induced gastric cancer (18). However, the source of the H$_2$O$_2$ was not determined. Recently, it has been demonstrated that in glutathione peroxidase 1 and 2 (Gpx1 and Gpx2) knockout mice, there is a high incidence of ileocolitis and microflora-associated cancers when mice are raised in conventional housing that includes infection with *Helicobacter* species (19), but when these mice are raised under germ-free conditions, they do not develop tumors. Because Gpx1 and Gpx2 are major detoxifying enzymes for H$_2$O$_2$, these results indicate that the...
reactive oxygen species responsible for the inflammation and carcinogenesis is bacterial infection-induced H$_2$O$_2$ production.

Here, we demonstrate that _H. pylori_ exposure leads to increased H$_2$O$_2$ production in AGS cells that can be inhibited by MDL72527, indicating that a polyamine oxidase activity is responsible for the H$_2$O$_2$ production. It should also be noted that our results are not restricted to a single cell line, because we have found similar effects of MDL 72527 in MKN-28 gastric epithelial stimulated with _H. pylori_ (data not shown). The SMO(PAOh1) activity is sufficient to produce DNA-damaging amounts of H$_2$O$_2$, as evidenced by both 8-oxoguanosine production and comet assay. Although generation of reactive oxygen species in response to _H. pylori_ has been previously linked to DNA damage (6, 7), our studies provide a new mechanism for generation of reactive oxygen species in epithelial cells and directly demonstrate that the polyamine oxidation causes both apoptosis and DNA damage by this mechanism. The DNA damage and apoptotic cell death produced by H$_2$O$_2$ was inhibited by MDL 72527, catalase, and most importantly, SMO(PAOh1)-specific small interfering RNA, thus demonstrating that the oxidase in question is, in fact, the spermine oxidase SMO(PAOh1) and not the classical N$^1$-acetyl-polyamine oxidase, PAO (16). These data combined with those from the transient transfection studies demonstrating that SMO(PAOh1) produces the same effects as _H. pylori_ exposure confirm that the oxidation of spermine by SMO(PAOh1) is the source of H$_2$O$_2$ in _H.
In summary, the results presented here demonstrate that *H. pylori* infection leads to the increased expression of an important polyamine catabolic enzyme, the polyamine oxidase, SMO(PAOh1). This enzyme oxidizes spermine producing the DNA-damaging reactive oxygen species, H₂O₂. Because reactive oxygen species have been directly linked to the etiology of multiple cancers including *H. pylori*-induced gastric cancer, these data are entirely consistent with the hypothesis that *H. pylori*-induced SMO(PAOh1) activity is responsible for the genotoxic insult that results in tumorigenic transformation of affected gastric epithelial cells.

### References

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