Induction of oxidative DNA damage by *Helicobacter pylori* in HT29 cells

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ABSTRACT

Infection with *Helicobacter pylori* has been associated with the development of gastric adenocarcinoma in humans. Several routes have been implicated, the main one being oxidative DNA damage resulting from chronic inflammation, which accompanies infection. However, DNA has been demonstrated in human cells after *in vitro* incubation with *H. pylori* sonicates. Using the fragment length analysis using restriction enzymes (FLARE) assay, this study investigates the DNA damaging potential of three clinical isolates of *H. pylori* on cultured HT29 cells. Significant amounts of oxidative DNA damage were detected in HT29 cells following a 72-hour incubation with each *H. pylori* isolate. As tumour induction is a known consequence of oxidative DNA damage, chronic infection with the organism may lead to the development of adenocarcinoma of the stomach.

KEY WORDS: DNA damage.
FLARE assay.
Helicobacter pylori.
Oxidative stress.

Introduction

Infection with the Gram-negative spiral organism *Helicobacter pylori* is well known to infect chronically up to 50% of the world's population.[1] Infection is always followed by chronic gastric inflammation,[2,3] which is usually asymptomatic. Duodenal and gastric ulceration occurs in approximately 15% of infected individuals.[4]

It is recognised that there are several aetiologies for gastric cancer, with one of the predominant causes being infection with *H. pylori*.[5] Of the patients infected chronically with *H. pylori*, approximately 1% develop either gastric carcinoma or mucosa-associated lymphoma.[6]

Early epidemiological studies implicate the organism in the development of gastric adenocarcinoma,[7,8] and many attempts have been undertaken to determine the pathophysiology of *H. pylori*.[9] Carcinogenesis in *H. pylori* infection has been attributed to several potential candidates, including an increase in epithelial cell proliferation,[10] a suppression of epithelial cell apoptosis,[11] and oxidative damage arising from chronic immune infiltration.[12]

Recently, however, research has demonstrated DNA damage in gastric mucous epithelial cells[6,13] and mouse lymphoma cells[14] following *in vitro* incubation with *H. pylori* sonicates. Bagchi and co-workers[15] also demonstrated the synthesis of reactive oxygen species (ROS) in gastric cells following *in vitro* incubation with live *H. pylori*. It is the production of these ROS and reactive nitrogen species that may play a role in the modification to DNA bases that can be a marker of oxidative DNA damage, and may lead to inflammation-mediated carcinogenesis.[16] Studies demonstrate that the *cag*A and VacA toxins of *H. pylori* are associated with cellular damage and alterations to the cell cycle.[1]

Following these observations, this study aims to assess the ability of three toxin-producing clinical isolates of *H. pylori* to induce oxidative DNA damage in colon adenocarcinoma cell lines utilising fragment length analysis using restriction enzymes (FLARE) assays.

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Materials and methods

Cell culture

A colon adenocarcinoma cell line (HT29; ECACC), a robust model of mammalian cell response to *H. pylori* infection, was cultured in Minimum Essential Medium (Gibco) supplemented with 10% fetal bovine serum (Gibco), 2 mmol/L L-glutamine (Gibco) and 1% non-essential amino acids (Gibco) at 37°C in 5% CO_2. Use of a primary cell line was discounted due to survival issues following exposure to *H. pylori* strains.

Bacterial culture

The *cag*A+ve and VacA-producing *H. pylori* strains J238 (*cag*A+ve, VacA s1/m1), J223 (cagA+ve, VacA s1/m2), both of which were clinical isolates from a patient with gastritis, and A101 (*cag*A+ve, VacA s1/m2), a clinical isolate from a patient with non-ulcer dyspepsia, were cultured and maintained in brain-heart infusion broth (Oxoid) supplemented with 10% laked horse blood (Oxoid). Clinical isolates of *Campylobacter jejuni* were grown under similar culture conditions to act as negative controls. Inoculated cultures were incubated in a microaerobic environment using a gas jar and Campypacks (Oxoid) at 37°C.

Cell/bacteria co-culture

HT29 cells were seeded at a concentration of 2 x 10^5 cells/mL in 12-well microtitre plates (Falcon) and left to adhere overnight. Harvested *H. pylori* strains were washed and

resuspended in phosphate-buffered saline (PBS) to give a suspension of 6 x 10^8 colony-forming units (cfu)/mL. Suspensions were added to HT29 cells at a final concentration of 6 x 10^7 cfu/mL and incubated for 72 h at 37°C in 5% CO_2.

In vitro *micronucleus test using the FLARE assay*

The protocol followed was adapted from those used by Trevigen Inc. and Collins *et al*.[17] Following a 72 h incubation, cells were trypsinised and washed once in PBS. After removing as much supernatant as possible, the cell pellet was vortex-mixed. Samples (400 µL) of 1% molten low-melting-point (LMP) agarose (Sigma) at 42°C were mixed with 10 µL of each cell suspension. Duplicates of the cell/agarose suspension were then run evenly down a microscope slide precoated with a thin layer of 1% normal-melting-point (NMP) agarose (Sigma). Slides were refrigerated for 5 min to allow the agarose to solidify before immersing in lysis solution for 2 h at 4°C.

Following removal from the lysis solution, the slides were washed (x3, each wash 5 min) with FLARE buffer at 4°C. Formamidopyrimidine-DNA-glycolase (Fpg; Trevigen Inc.) was diluted 1 in 50 in enzyme reaction buffer, and 32-µL volumes (containing 2 units of protein) were added to each slide. Control slides were treated with an equal volume of reaction buffer only. After applying coverslips, slides were incubated in a moist box for 30 min at 37°C. Coverslips were then removed and the slides placed in an electrophoresis subtank containing electrophoresis solution at 4°C. The DNA was left to uncoil for 40 min.

The electrophoresis module was packed in ice during uncoiling and electrophoresis, in order to optimise sample adherence and decrease background damage (Trevigen protocol). Then, 25 V was applied for 45 min. Slides were then washed (x3, each wash 5 min) in neutralisation buffer at 4°C, flooded with 70% ethanol for 5 min, and allowed to dry in the air. Ethidium bromide (50 µL, 5 µg/mL) was pippetted onto each slide and a coverslip applied to ensure even coverage.

Slides were stained for 10 min before destaining in PBS washes (x3, each was 5 min). New coverslips were applied. Viewing was carried out using a fluorescence microscope (Zeiss-Axioskop, 100 W mercury lamp) with integral digital camera (Nikon Dn100) and imaging software (Nikon, Eclipse-net). Tail length was used as a measure of DNA damage.

ImageJ (freeware, available from http://rsbweb.nih.gov/ij) was used to score the gels. This program permits conversion of the image to binary information, in order to define more clearly the threshold of fluorescence.

Table 1. The *t*-values for the tail lengths of HT29 cells with and without Fpg treatment following incubation with all three strains when compared to un-infected controls.

H. pylori strain	Fpg-treated	Significant at $P \leq 0.05$	Untreated	Significant at $P \leq 0.05$
J238	5.98	Yes	2.21	No
A101	12.16	Yes	0.45	No
J233	5.08	Yes	0.45	No

Statistical analysis

Groups of 100 cells were analysed (x400 magnification) for each triplicate slide. Fpg-treated and -untreated cells from triplicate *H. pylori* J238 infected cultures were compared using Student's *t*-test, and mean tail length was found to be significantly different. Therefore, a hydrogen peroxide positive control was deemed unnecessary, as activity of the Fpg enzyme was demonstrated.

The *t*-test was also used to compare tail length from the following: non-Fpg-treated, infected and uninfected samples, to determine any significant difference in strand break levels without the presence of Fpg; and tail length of Fpg-treated, infected and non-infected samples, to determine any significant difference in oxidative damage levels.

Results were considered significant at $P \leq 0.05$.

Results

The FLARE assay was used to assess DNA damage levels in the HT29 gastric cells following incubation with *H. pylori*. In addition, this assay permitted assessment of the proportion of oxidative damage. The genotoxicity of three CagA- and VacA-producing clinical isolates of *H. pylori* (strains J238, A101 and J233) was assessed in two individual, duplicate experiments after a 72 h incubation with HT29 cells.

To determine the significance of difference in tail length between Fpg-treated and -untreated cells, triplicate cultures of HT29 cells infected with *H. pylori* J238 were assayed, the data were pooled and then Student's *t*-test was applied to the resulting tail lengths (Table 1). The mean tail length of Fpg-treated cells was found to be significantly higher than

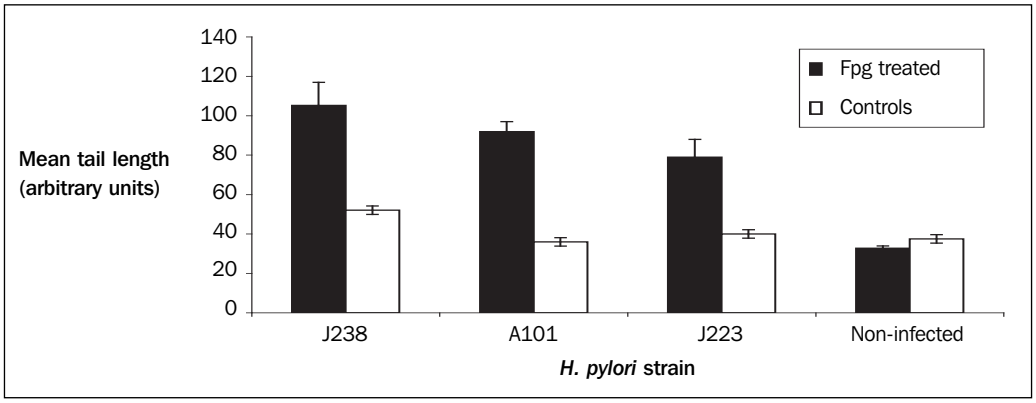

Fig. 1. Mean tail length of comets (as a measure of DNA damage) after incubation of HT29 cells with *H. pylori* strains J238, A101 and J233, with and without Fpg treatment. Results shown are the mean of three separate cultures, with 100 cells/culture ± SEM. Results are representative of two individual experiments.

that of non-treated cells (at ∞ degrees of freedom, $P \leq 0.001$). This result demonstrates the activity of the Fpg used in this study.

Following a 72 h incubation of HT29 cells with three CagA- and VacA- producing clinical isolates of *H. pylori* and subsequent treatment with Fpg, DNA damage was observed at significantly higher levels than was present in uninfected controls (Fig. 1). Measurement of the mean tail length of infected cells when treated with enzyme buffer alone was not found to be significantly different from that of non-infected cells (Fig. 1).

The significance of these results was tested using Student's *t*-test. Comet tail length after incubation of HT29 cells with strains J238, A101 and J223 was found to be significant at $P \leq 0.05$ (Table 1) when compared to controls.

Discussion

The alkaline single-cell gel electrophoresis assay is a highly sensitive detector of single and double DNA strand breaks, even when present at very low levels.[17] With the inclusion of Fpg, a molecule that cleaves DNA at sites of oxidative adducts (e.g., 8-oxo-deoxyguanosine [8-oxo-dG]), this study demonstrated a highly significant increase in damage in *H. pylori*-infected cells compared to control cells after a 72 h incubation. Without Fpg, no significant damage was detected, suggesting that the majority of the damage present was due to the formation and subsequent removal of oxidative adducts.

Oxidative stress is considered to be the most likely mechanism for the induction of tumour growth in the stomach of infected individuals,[18] as *H. pylori* is chronically, but largely unsuccessfully, attacked by the immune system.[19] The present study shows, however, that *H. pylori* has the ability to cause a significant increase in oxidative DNA damage in a human gastrointestinal cell line, without the involvement of immune cells. This effect may play a central role in tumour induction.

Oxidative adducts in mammalian cell DNA are removed via a base excision repair pathway[20] by DNA glycosylases homologous to the *Escherichia coli*-derived Fpg enzyme used in this study. Without the addition of repair enzymes, the comet assay can only detect single-strand breaks that have occurred transiently as a result of this pathway. This explains the lack of any significant increase in tail length between infected and non-infected cells. The addition of large amounts of Fpg to the cells permitted any oxidative adducts present to be converted to strand breaks, which then could be visualised in the FLARE assay.

The most abundant and mutagenic oxidative adduct is 8-oxo-dG.[21] If DNA replication occurs prior to the repair of this adduct, a GC↔TA mutagenic transversion often results.[22] GC↔TA transversions frequently have been detected in the *p53* gene and the *ras* proto-oncogene in some primary tumours.[23] Both genes are known hotspots for free-radical damage.[24] It has been shown that *p53* is a specific target for oxidative DNA damage in *H. pylori*-positive biopsy samples.[25]

A possible contributing factor to this oxidative damage is the large amount of superoxide (O_2^-) that *H. pylori* generates.[26] This has a protective effect on the bacterium *in vivo*, as it reacts with and thus deactivates the bactericidal nitric oxide (NO) present in gastric juice. The product of this reaction is peroxynitrite ($ONOO^-$), a further metabolite of which is the hydroxyl radical (OH^-). These highly cytotoxic molecules, along with superoxide itself, may be responsible for a large amount of the oxidative damage seen in *H. pylori*-infected gastric epithelia from biopsy samples.[27]

Another explanation is that the gastric cells produce ROS in response to *H. pylori* infection. This effect has been studied *in vitro* by Bagchi *et al*.,[15] who reported enhanced production of ROS in gastric cells after incubation with *H. pylori*. Following this, O'Rourke *et al*.[28] used endonuclease III knockout mutants of *H. pylori*, which have an impaired ability to cleave DNA at internal sites and thus repair oxidative DNA damage, to show that, in response to infection, host cells can induce lethal oxidative adducts in the *H. pylori* genome. Other studies also show the importance of *H. pylori*, when damage is directed towards epithelial cells and leucocytes.[29–31]

In an *in vitro* study such as the one reported here, oxidative damage to the DNA of infected gastric cells may be attributed largely to the superoxide radicals generated by *H. pylori,* meant to aid the organism's survival in the stomach, and to the ROS generated by the gastric cells as a protective response against the bacteria. With regard to the latter, the investigation of other enteric bacteria is important in order to elucidate whether or not ROS are produced generally by gastric cells in response to infection, and, if so, what potential this effect has in inducing DNA damage.

Human cells contain several natural antioxidants that are the cell's main defence against ROS. Glutathione (GSH), a major cellular antioxidant, is transcribed continuously at a basal level.[32] Although an up-regulation of transcription can be sustained temporarily in response to high levels of ROS within the cell,[33] this cannot continue indefinitely. Thus, chronic attack by large numbers of these molecules eventually may deplete GSH levels and overwhelm the cell's natural antioxidant defence.[34] Glutathione is largely depleted in *H. pylori*-infected cultured gastric epithelial cells.[35] In addition, *H. pylori* infection has been associated with a significant reduction in ascorbic acid in gastric juice.[36]

Although the persistent direct formation of ROS in gastric cells by *H. pylori* may be a major route to the induction of gastric tumours, this undoubtedly is a multifactoral process. The immune response to infection also contributes to the amount of ROS with which the infected gastric cells are challenged, and the accompanying depletion of the cell's intrinsic antioxidant defence system, and one of the major dietary antioxidants, can only augment the resulting damage.

At present, no screening process is in place to detect *H. pylori* carriers. Presence of the organism is only investigated by serology or the urea breath test if a patient presents with gastritis or peptic ulcer disease, with antimicrobials then used to eradicate the infection.

The results of the present study highlight the importance of antioxidants as a dietary supplement for the general population, and also substantiate the need for free-radical scavengers as adjuvant therapy in the eradication of *H. pylori* infection. □

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