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Helicobacter pylori γ-Glutamyltranspeptidase Induces Cell Cycle Arrest at the G1-S Phase Transition

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In our previous study, we showed that *Helicobacter pylori* γ-glutamyltranspeptidase (GGT) is associated with *H. pylori*-induced apoptosis through a mitochondrial pathway. To better understand the role of GGT in apoptosis, we examined the effect of GGT on cell cycle regulation in AGS cells. To determine the effect of recombinant GGT (rGGT) on cell cycle distribution and apoptosis, rGGT-treated and untreated AGS cells were analyzed in parallel by flow cytometry using propidium iodide (PI). We found that rGGT inhibited the growth of AGS cells in a time-dependent manner, and that the pre-exposure of cells to a caspase-3 inhibitor (z-DEVD-fmk) effectively blocked GGT-induced apoptosis. Cell cycle analysis showed G1 phase arrest and apoptosis in AGS cells following rGGT treatment. The rGGT-mediated G1 phase arrest was found to be associated with down-regulation of cyclin E, cyclin A, Cdk 4, and Cdk 6, and the up-regulation of the cyclin-dependent kinase (Cdk) inhibitors p27 and p21. Our results suggest that *H. pylori* GGT induces cell cycle arrest at the G1-S phase transition.

***Keywords*:** *H. pylori*, γ-glutamyltranspeptidase, cell cycle

Helicobacter pylori is associated with the progression of gastric cancer (Rhee *et al*., 1990; Banatvala *et al*., 1993; Baik *et al*., 1996). *H. pylori* infection also induces apoptosis in gastric epithelial cells (Moss, 1998), which has been suggested to play a significant role in the pathological events associated with infection (Correa and Miller, 1998).

The maintenance of homeostasis in normal tissues reflects a balance between cell proliferation and death. This balance is often disrupted in tumor cells, which have a high rate of proliferation. Anti-tumor effects are often the result of biochemical alterations in specific functional pathways, such as inhibition of proliferation, growth arrest at one or more cell cycle checkpoints, enhanced apoptosis, or the modulation of key signal transduction pathways that control the expression of important regulatory enzymes (Grana and Reddy, 1995; Swanton, 2004).

Cyclin-dependent kinases (Cdks), Cdk inhibitors, and cyclins are important regulators of cell cycle progression. Each phase of the cell cycle is governed by different Cdks in association with specific cyclins, which function as regulatory subunits. For example, the G1 phase is controlled by Cdk 4 and Cdk 6, which are associated with cyclin D; the transition from late G1 to early S is controlled by Cdk 2 and cyclin E; the S phase is regulated by Cdk 2 and cyclin A; and the G2/M checkpoint is regulated by Cdc2 kinase (Cdk 1) and cyclin A/cyclin B (Dulic *et al*., 1992; MacLachlan *et al*., 1995; Vermeulen *et al*., 2003).

The cellular levels of Cdk inhibitors also play a key role in the regulation of cell cycle progression. The Cip/Kip family of Cdk inhibitors, which includes p21/WAF1 and p27/KIP, bind to cyclin/Cdk complexes and prevent kinase activation, thereby blocking the progression of the cell cycle at G0/G1 or G2/M (Elledges and Harper, 1994; Sandal, 2002). High expression levels of Cdks and cyclins and the accompanying increase in Cdk activity are observed in most cancer cells: these changes likely cause the deregulation of the cell cycle and unchecked cell proliferation (Hall and Peters, 1996).

Previously, we showed that gastric epithelial cells undergo apoptosis when cultured *in vitro* with rGGT (Kim *et al*., 2007). Here, we investigate the mechanism of apoptosis by rGGT in epithelial cells by examining the effects of rGGT on cell cycle progression and measuring the expression levels of the following cell cycle regulatory proteins: cyclin A, D, and E; Cdk 4 and 6; and the Cdk inhibitors p27 and p21.

Materials and Methods

Bacterial strain and culture conditions

H. pylori strain 51 was incubated on brucella agar plates containing 10% bovine serum. Bacterial cells were cultivated overnight at 37°C in an atmosphere of 10% CO_2 and 100% humidity. *H. pylori* strain 51 was deposited in and supplied from the *H. pylori* Korean Type Culture Collection (Gyeongsang National University, Korea).

Cell culture

AGS cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 100 units/ml gentamicin, and incubated in a humidified atmosphere of 5% CO_2 at 37°C. For

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experiments with synchronized cells, AGS cells were serum-deprived for 48 h.

Viability assay

The effect of rGGT (50 ng/ml) on the viability of AGS cells was determined with the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide) assay. The cells were plated at 1×10^4 cells per well in 100 μl of complete culture medium and treated with rGGT (50 ng/ml) for 6, 12, and 24 h at 37°C in a humidified incubator, either with or without 30 min pretreatment with 100 μM caspase-3 specific inhibitor z-DEVD-fmk (Santa Cruz Biotechnology, USA) in 96-well microtiter plates. After treating the cells with rGGT, MTT (5 mg/ml in phosphate buffered saline) was added to each well and incubated for 4 h. After careful removal of the medium, 100 μl DMSO was added to each well, and the plates were shaken. Absorbance was recorded on a microplate reader at a wavelength of 540 nm. The effect of rGGT on growth inhibition was assessed as percent cell viability, where cells with no treatment were considered 100% viable. The number of surviving cells was enumerated using trypan blue dye. We used adherent cells only directly for viability assay.

The expression and purification of rGGT was carried out as previously described (Kim *et al*., 2007).

Nuclear staining with DAPI

After treating the cells with rGGT (50 ng/ml) for 6, 12, and 24 h, the harvested cells were washed in ice-cold PBS, and fixed with 70% ethanol in PBS for 10 min at room temperature. The fixed cells were washed with PBS and stained with 2 μg/ml of 4, 6-diamidino-2-phenylindole (DAPI, Sigma, USA) dissolved in PBS for 10 min at room temperature. We used adherent cells only directly for fluorescence microscopy.

The nuclear morphology of the cells was examined by fluorescence microscopy (Olympus, Japan). DAPI permeates the plasma membrane and yields blue chromatin. Viable cells display normal nuclear size and blue fluorescence, whereas apoptotic cells show condensed chromatin and fragmented nuclei.

Flow cytometry analysis

Following treatment, adherent and floating cells were harvested by using trypsin and washed once with phosphate-buffered saline (PBS), fixed in ice-cold 70% ethanol, and stored at 4°C. Cell pellets were suspended in 400 μl of a 0.2 mg/ml propidium iodide solution (PI, Sigma) containing 2 mg/ml RNase (Sigma Chemical Co., USA), and then incubated in the dark at room temperature for 30 min. Data acquisition and analysis were performed using a FACScan system equipped with FACSCalibur (Becton Dickinson Biosciences, USA). Debris was eliminated from the analysis using a forward angle light scatter threshold. Cell doublets and other clumps were eliminated from the analysis by gating the fluorescence pulse area (integral fluorescence). All fluorescence and laser light scatter measurements were carried out with linear signal-processing electronics. At least 10,000 cells were analyzed for each condition, and all experiments were independently performed more than three times.

Western blot analysis

Following treatment with rGGT, cells were collected and suspended in lysis buffer (PRO-PREP, Intron Biotechnology, Korea). Cell lysates were subjected to centrifugation, and protein concentrations were determined by the Lowery method (Lowry *et al*., 1951) using bovine serum albumin as a standard. Lysates containing 20 μg of protein were subjected to 12.5% SDS-polyacrylamide gel electrophoresis (PAGE), and then the proteins were electrophoretically transferred onto a nitrocellulose transfer membrane (PROTRAN®, Schleicher & Schuell, Germany) in blotting buffer (39 mM glycine, 48 mM Tris base, 20% methanol, and 0.037% SDS). The transfer conditions were as follows: 20 volts constant for 40 min in a Trans blot SD semi-dry Transfer Cell (Bio-Rad, USA). Nonspecific binding sites were blocked by incubation with 1% BSA in TBS-T (25 mmol/L Tris-HCl, 137 mmol/L NaCl, 137 mmol/L KCl, 0.1% Tween 20, pH 7.4) for 1 h at room temperature with gentle shaking. The nitrocellulose membranes were then incubated with primary antibodies against cyclin A, cyclin E (Santa Cruz), p27 (sc-528-G, Santa Cruz), p21 (sc-397-G, Santa Cruz), Cdk 4 or Cdk 6 (Santa Cruz) in TBS-T at 4°C overnight. Membranes were washed with TBS-T and then incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit or donkey anti-goat IgG secondary antibodies (1:5,000, Santa Cruz). The membranes were washed extensively with TBS-T, and then immunoreactive proteins were visualized using enhanced chemiluminescence (ECL, Amersham, UK) and autoradiography.

Results

Inhibition of cell viability and induction of apoptosis by rGGT

AGS cells at approximately 70% confluence were incubated with rGGT for 6, 12, and 24 h in complete medium containing 10% FBS. After 24 h, rGGT-treated cells showed a significant decrease in cell number compared with cells pretreated with a caspase-3 inhibitor in response to rGGT exposure (Fig. 1A). Morphological analysis with DAPI staining showed nuclei with chromatin condensation and time-dependent formation of apoptotic bodies in the cells cultured with rGGT. On the other hand, very few apoptotic bodies were observed in the culture pretreated with the caspase-3 inhibitor (Fig. 1C). The degree of apoptosis was quantified by analyzing the amount of sub G1 DNA via flow cytometry; pre-exposure of cells to a caspase-3 inhibitor (z-DEVD-fmk) effectively blocked GGT-induced apoptosis (Fig. 1B).

Cell cycle arrest in G1 by rGGT

To determine the effect of rGGT on cell cycle distribution, untreated and rGGT-treated AGS cells were analyzed in parallel. AGS cells were synchronized by serum-deprivation for 48 h. Serum was then added back to the culture media with or without rGGT, and cell cycle progression was analyzed with a FACScan flow cytometer. Compared to control cells treated with serum alone, treatment with serum plus rGGT for 12 h inhibited cell cycle progression at G1 (Fig. 2A), and inhibited the increase in cells in S phase (Fig. 2B) and G2/M phase (Fig. 2C). These results indicate that rGGT inhibits cell cycle progression from G1 to S phase. The inhibition of the G1 to S phase transition by rGGT was also observed in exponentially growing (non-serum-deprived) AGS cells and in cells that were 100% confluent; however, under both of these conditions, the changes were less marked than in serum-deprived cells (data not shown).

Expression of cell cycle-related proteins

Western blot analysis of cell cycle regulatory proteins was carried out following the stimulation of synchronized (serum-

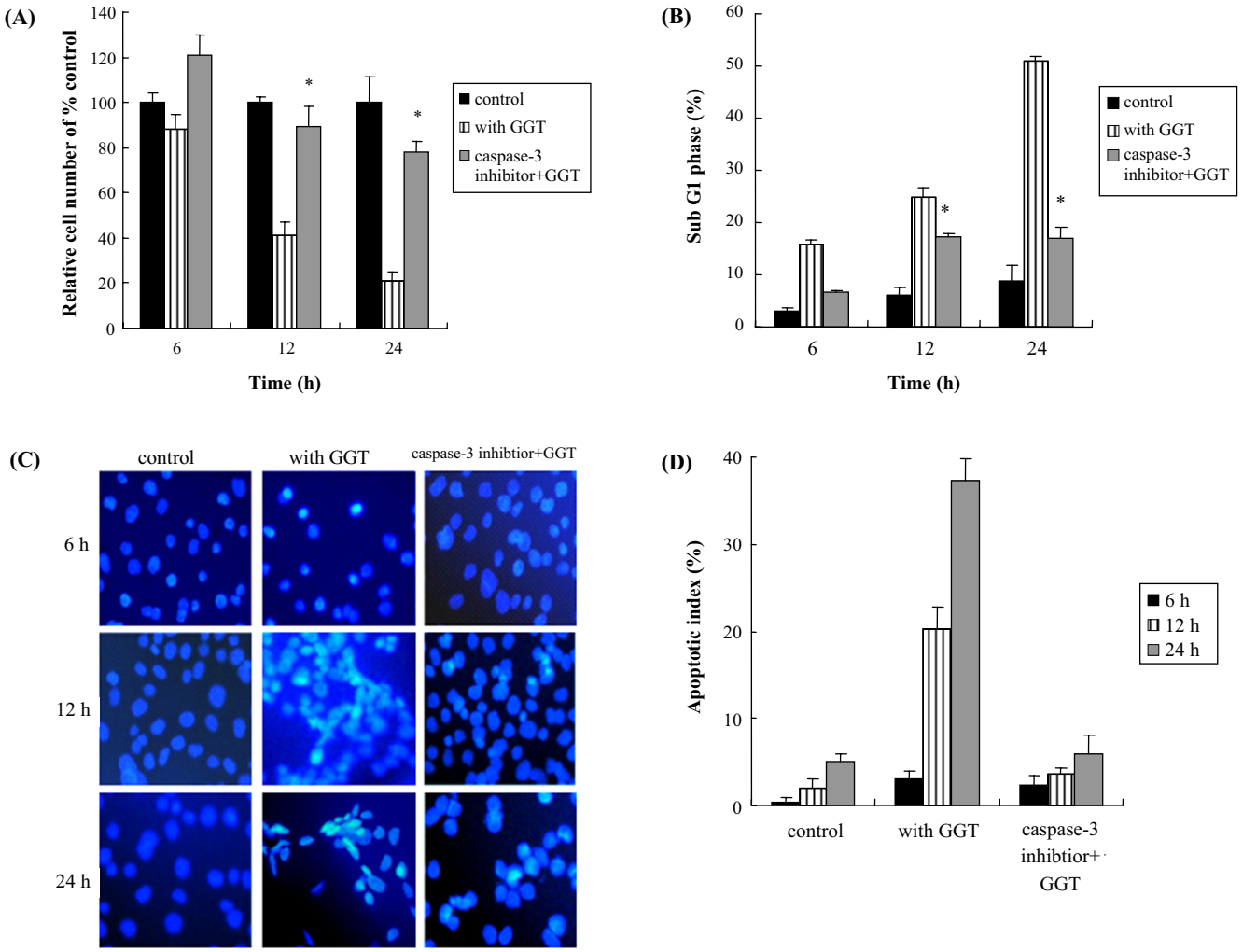

Fig. 1. Growth inhibition and apoptosis induction of AGS cells following treatment with 50 ng/ml rGGT for 6, 12, and 24 h. The cells were pretreated for 30 min with a caspase-3 inhibitor (z-DEVD-fmk) and then exposed to rGGT for 24 h. Control cells were treated with buffer without rGGT. (A) The number of cells was estimated by trypan blue dye. The data shown are the Mean±SD of three independent experiments. (B) After 24 h incubation with rGGT, the cells were collected and stained with PI for flow cytometry analysis. The percentage of cells with hypodiploid DNA content represents the fraction undergoing apoptotic DNA degradation. The data shown are the Mean±SD of three independent experiments. (C) The cells were sampled, fixed and stained with DAPI. The stained nuclei were then observed under a fluorescent microscope using a blue filter. (D) The percentage of apoptotic cells in each time was determined by DAPI staning. Magnification, ×400.
***** $p<0.05$ when compared with the rGGT treated group.

deprived) AGS cells with serum plus rGGT. The levels of cyclin E and cyclin A decreased 12-24 h after serum stimulition, reflecting the progression of these cells from G1 into early S phase. The expression of Cdk 4 and Cdk 6 also decreased in a time-dependent manner. The expression of the Cdk inhibitors p21 and p27 increased at 12-24 h after serum-stimulation in the presence of rGGT (Fig. 3).

Discussion

H. pylori GGT is an enzyme that utilizes extracellular glutamine and glutathione as a source of glutamate, which may play important role in damaging of mammalian cells and the pathophysiology of *H. pylori* (Shibayama *et al*., 2007). *H. pylori* GGT is likely associated with the cell membrane, which would explain the observation that direct contact of *H. pylori* with host cells is required for the induction of apoptosis (Shibayama *et al*., 2001; Kim *et al*., 2008). Previously, we also demonstrated that GGT is bacterial virulence factor responsible for *H. pylori* related apoptosis by mitochondrial pathway (Kim *et al*., 2007). On the other hand, it was reported that GGT induce upregulation of COX-2 expression in *H. pylori*-associated gastric carcinogenesis (Busiello *et al*., 2004). In addition, *H. pylori* virulence factor GGT might contribute to gastric carcinogenesis by a chronic inflammatory response, or by regulation of proliferation and/or survival that control epithelial cell homeostasis (Romano *et al*., 2006).

In our study, cell cycle analysis showed that exposure of

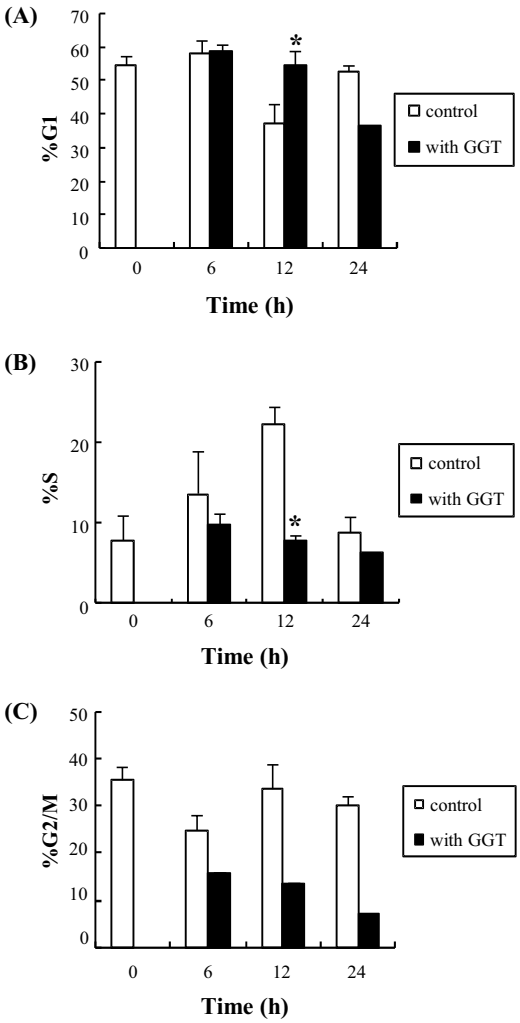

Fig. 2. Cell cycle distribution of AGS cells incubated in cell medium alone (control) or cell medium containing rGGT (50 ng/ml cell medium) at 0, 6, 12, and 24 h. The percentage of cells in each phase was determined by DNA staining with PI and flow cytometry. (A) G1 phase. (B) S phase. (C) G2/M phase. * $p<0.05$ when compared with the control group.

gastric epithelial cells to *H. pylori* GGT inhibited cell cycle progression at the G1 to S phase transition, and also altered the expression of specific cell cycle regulatory proteins. It seems likely, therefore, that these effects and the associated induction of apoptosis are responsible for the inhibition of gastric epithelial cell growth by *H. pylori* GGT that we observed in the current study and as well as previous work (Shibayama *et al*., 2001; Kim *et al*., 2007). The effects of GGT on the cell cycle were most pronounced when we used cell cultures that were partially synchronized in G0/G1 by serum deprivation, and then stimulated to progress through G1 to S by the addition of serum. Using this protocol, we observed changes in the expression of specific cell cycle regulatory proteins in cells that were treated with rGGT. It is known that deregulation of cell cycle-related proteins such as Cdks and Cdk inhibitors alter critical events in the cell cycle (Vermeulen *et al*., 2003).

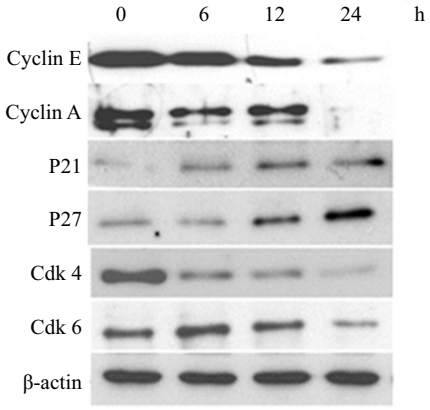

Fig. 3. Western blot analysis of cell cycle-related proteins induced by *H. pylori* rGGT in AGS cells. After treatment with 50 ng/ml rGGT for 0, 6, 12, and 24 h, cell lysates were prepared and subjected to western blot analysis using antibodies for cyclin E, cyclin A, p21, p27, Cdk 4, and Cdk 6. The decrease in cyclin E, cyclin A, Cdk 4, and Cdk 6 and the increase in p21 and p27 in cells treated with rGGT is shown. β-Actin was used as a loading control.

We propose that *H. pylori* GGT enhance apoptosis through its ability to deregulate cell-cycle progression. The effects of rGGT on the cell cycle were also observed in exponentially growing cells, but were less pronounced than in serum-deprived cells. These results suggest that the effects of rGGT are somewhat dependent on the growth characteristics of the target cells. There have been relatively few studies examining the effects of bacteria on the cell cycle in epithelial cells. It has been shown that *H. pylori* cag PAI accelerated the progression of the cell cycle from G1 into G2/M in AGS cells at 6 h (Peek *et al*., 1999).

In previous study, the ability of GGT to induce apoptosis in AGS cells was investigated. Treatment of the rGGT resulted in sequences of events marked by apoptosis, as shown by loss of cell viability, and morphology change. rGGT-induced apoptotic cell death that is associated with cytochrome *c* translocation, caspase-3 and -9 activation, dysregulation of Bcl-2 and Bax. Our study was investigated that rGGT inhibits cell proliferation by arresting AGS cells in the G1 phase. rGGT treatment showed morphological changes with chromatin condensation in AGS cells and pre-exposure of cells to a caspase-3 inhibitor (z-DEVD-fmk) effectively blocked GGT-induced apoptosis (Fig. 1B). In many cases, apoptosis induced by *H. pylori* is dependent on alteration of the cell cycle, which is controlled through several different Cdk regulatory mechanisms (Draetta, 1990; Morgan, 1995).

H. pylori infection of gastric epithelial cells is associated with growth inhibition (Fan *et al*., 1995; Wagner *et al*., 1997; Shirin *et al*., 1999) followed by cell cycle arrest at both G1-S and G2-M phases (Lew *et al*., 1991; Parker *et al*., 1995; Ahmed *et al*., 2000; Chiou *et al*., 2003).

In the present study, the cell cycle distribution by rGGT on AGS cells were investigated. We found that rGGT inhibits the growth of AGS cells in a time-dependent manner, with subsequent accumulation of subG1 phase cells. Compared to control cells, rGGT-treated cells for 12 h inhibited cell cycle progression at G1 phase and inhibited the increase in cells in

S and G2/M phase (Fig. 2). The decrease of G1 phase cells in rGGT-treated cells at 24 h seems to be related to the increase of sub G1 phase cells in the same group as shown in Fig. 1.

The exposure of AGS cells to rGGT was associated with a reduction in the expression of cyclin A and cyclin E. Further, rGGT exposure increased levels of p27 and p21 (Fig. 3), which bind to and inhibit several cyclin/Cdk complexes, particularly cyclin E/Cdk 2; these complexes are essential in regulating passage into mitosis. Both of these effects are consistent with the inhibition of cell cycle progression and entry into S phase as well as inhibition of the G1 to S transition. D-type cyclins form complexes with CDK4 and CDK6 and the CDK4/CDK6 complex appears to be necessary for transition through G1 phase (Sherr, 1994; Ohtsubo *et al*., 1995). In our study, we investigated the change of the CDK4/CDK6, the expression of CDK4/CDK6 was decreased in accordance with *H. pylori* GGT treatment. In another report, it was demonstrated that the expression of cyclin D increased in MKN45 cells by *H. pylori* but not in AGS cells (Ding *et al*., 2008). Thus, our results suggest that *H. pylori* GGT acts as a "brake" at the G1 to S phase transition, thereby disrupting the normal function of several components of the cell cycle.

Other works have shown that bacteria such as *Haemophilus*, *Escherichia coli*, and *Campylobacter* can block epithelial cell division either at the G1/S or G2/M phase of the cell cycle (Peres *et al*., 1997; Whitehouse *et al*., 1998; Cortes *et al*., 1999). Thus, microorganisms seem to have evolved a number of different mechanisms to inhibit the mammalian cell cycle, which may partly explain their specific pathogenic effects.

Further exploration of the molecular mechanisms underlying the effects of *H. pylori* GGT on cell cycle control may provide insight into the role of this organism in gastric carcinogenesis.

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