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Effects of *Helicobacter pylori* on biological characteristics of gastric epithelial cells

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Summary. Infection with *Helicobacter pylori (H. pylori)* strains is linked to an increased risk of inflammation and gastric cancer. To investigate the effects of *H. pylori* on biological characteristics of gastric epithelial cells SGC-7901, derived from human adenocarcinoma, morphological appearances of both the pathogen and these cells, as well as features of attachment and internalization were observed by using transmission electron microscopy (TEM). We also investigated cell junctions and invasion by TEM and Transwell Invasion Assay. Cell proliferation and apoptosis were assessed by using chromogenic methylthiazol tetrazolium bromide (MTT) dye and flow cytometry. Three types of *H. pylori* were observed around, attaching to, or invading tumor cells. Cellular damage was characterized by vacuolar degeneration, dilated endoplasmic reticulum (ER), and reduction of organelles. Cell junctions and cell microvilli reduced or disappeared. *H. pylori* inhibited cell proliferation, whereas it had no effect on apoptosis. It also promoted gastric carcinoma cell invasion. *H. pylori* damages cell construction, destroys cell junctions, inhibits cell proliferation, promotes cell invasive ability, and, therefore, might accelerate the malignant progress and metastasis of gastric cancer.

Key words: *Helicobacter pylori*, Gastric carcinoma, Invasion, Ultrastructure, Apoptosis, Proliferation

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Introduction

Gastric carcinoma is the fourth most common cancer type and the second cause of cancer-related death worldwide (Konturek et al., 2006). Although advanced surgical techniques and adjuvant therapies have proved useful in tumor treatment, metastasis remains a major cause for poor prognosis and death. *H. pylori* infection is an established risk factor for gastric cancer development (Tomb et al., 1997; Lu et al., 2005), but the exact underlying mechanism still remains obscure. The effects of *H. pylori* infection on gastric epithelial cells have been extensively studied. The most important phenomena observed under the light and electron microscope are that there were lots of small or large vacuoles in the cytoplasm (Cover and Blanke, 2005; Chiozzi et al., 2009) which had been derived from the fusion and/or swelling of membrane-bound compartments or autophagic vacuoles (Terebiznik et al., 2009).

Cell junctions include tight junction, gap junction, chemical synapse, desmosome, and so on. *H. pylori* CagA gene induced damage to the cell junction of gastric epithelial cells (Amieva et al., 2003). Xu et al. (2009) found that *H. pylori* reduced the number of cell gap junctions, the number of junctions/ unit perimeter, the length of junctions/unit perimeter, and increased the width of intercellular space. The inhibition of gap junctional intercellular communication (GJIC) gastric epithelial cells was associated with gastric cancer. Disruption of cell junction may decrease the cell GJIC function and result in occurrence and development of gastric cancer (Tao et al., 2007; Xu et al., 2009). This study used TEM to observe the effect of *H. pylori* on morphologic changes and cell junction ultrastructure of gastric epithelial cells.

Various mechanisms have been postulated about the relationships between *H. pylori* infection and gastric cancer. *H. pylori* regulates the inflammatory process and promotes gastric carcinogenesis through induction of gene mutations and protein modulation (Keates et al., 2007; Steele et al., 2007; Tomimori et al., 2007; Marcos et al., 2008; André et al., 2010), increased angiogenesis (Mangia et al., 2006; Yamac et al., 2008; Lin et al., 2010), and cross-talk with stem cells (Pilpilidis et al., 2011), thereby being involved in gastric oncogenesis. Some investigators reported that *H. pylori* induced apoptosis (Galmiche et al., 2000; Willhite et al., 2003; Liu et al., 2006; Chiozzi et al., 2009) and promoted proliferation (Galmiche et al., 2000; Kuck et al., 2001; Moss et al., 2001; Chen et al., 2006), whereas others believed that there were no effects on apoptosis or proliferation of gastric cancer cells (Chow et al., 1995; Cho et al., 2003; Cover et al., 2003; Tao et al., 2007; Wu et al., 2008; Oldani et al., 2009). The effects of *H. pylori* on motility and invasion were rarely studied (Zhang et al., 2007; Snider and Cardelli, 2009). In this situation, we try to elucidate whether *H. pylori* plays an important role in migrative and invasive properties of cancer cells.

Materials and methods

H. pylori strain culture

International standard *H. pylori* 26695 strain (wild-type) was kindly provided by Microbiology Department of School of Medicine, Shanghai TiaoTong University. The *H. pylori* strain was cultured on Brucella agar plates containing 10 % sheep blood supplemented with 10 mg/L vancomycin, 2500 U/L polymyxin B, and 5 mg/L trimethoprim, in an anerobic jar consisting of 50 mL/L O_2, 100 mL/L CO_2, and 850 mL/L N_2 at 37°C for 3 d in a humid condition. Helicobacter colonies were further identified by their typical morphology and characteristic appearance on Gram staining. Using cotton swabs, bacteria harvested from the plates were suspended in 200 ml of brain heart infusion broth containing 10 % fetal bovine serum (FBS) and then cultured in the liquid medium at 37°C for 2 days in a controlled micro-aerophilic environment. Bacteria were harvested from the broth culture by centrifugation and then resuspended at the concentrations indicated in antibiotic-free medium. Cultured bacteria reached a density of $3x10^9$ CFU/ml. All procedures were performed with the approval of the appropriate institutional biosafety review committees and in compliance with their guidelines for biohazards.

Cell culture

Human gastric epithelial cell line SGC-7901 used in the experiments was purchased from the Cell Bank of Shanghai Institute of Cell Biology of the Chinese Academy of Sciences. The cells were routinely cultured in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/L streptomycin, and 100 U/L penicillin in air with 5% CO_2 at 37°C.

Co-cultivation of gastric epithelial cell line SGC-7901 with H. pylori

SGC-7901 cells were prepared by seeding $2x10^6$ cells on plates. After overnight incubation, the medium was replaced with fresh medium without antibiotics. The *H. pylori* were harvested from plates by suspension in 2 ml PBS and washed twice with PBS. After centrifugation at 4000 r.p.m. for 5 min, the bacteria were resuspended in 2 ml PBS and immediately added to cell culture plates. The ratio of the number of bacteria to the number of cells were 10:1, 100:1 and 200:1. Gastric epithelial cells infected with *H. pylori* were cultured in air with 5% CO_2 at 37°C.

Transmission electronic microscope observation

SGC-7901 cells were grown to confluence on cover glasses in six-well plates, and *H. pylori* were added to each cover glass at a ratio of 100:1. The cultures were placed in air with 5% CO_2 at 37°C for 24 h in a total volume of 2 ml. Then, the cover glasses were removed and washed twice with PBS. The cells were fixed first with 2.5% glutaraldehyde for 2 h, and then with 2% osmic acid. The morphologic changes of *H. pylori* and SGC-7901 cells, as well as interaction between bacteria and cells were observed with a transmission electron microscope.

MTT assay

MTT assay was used to detect cell viability. SGC-7901 cells were seeded onto 96-well plates and incubated at 37°C for 24 h. The cells were then incubated or not with *H. pylori* (MOI: 100:1). After a further time in culture, 100 μL of MTT (5 mg/ml, Sigma) was added to each well and incubated again for 4 h at 37°C. MTT was then removed, and 150 μL of dimethylsulphoxide was added to each well. The absorbance values were measured at a wave length of 490 nm using an ELISA reader. Cell viability was calculated by survival ratio: (A value of treated group) / (A value of control group)x100 %. The inhibitory rates (IR) were calculated according to the following formula: IR = [1-(A value of treated group) / (A value of control group)]x100 %.

Apoptosis assay by flow cytometry

About $1.0x10^6$ cells infected with *H. pylori* were harvested by trypsin digestion and centrifugation (at 1000 r.p.m. for 5 min), then washed twice with PBS. Then, the cells were fixed with 70% ethanol. The

apoptosis of SGC-7901 cells was determined by flow cytometry.

Transwell and invasion assays

For transwell chamber-based invasion assays, equal amounts of cells were loaded into an insert provided with serum-free medium and allowed to pass through an 8-Ìm-pore polycarbonate filter, which had been either pre-coated with 100 μg of Matrigel (Becton Dickinson, San Jose, CA) for invasion assay or left uncoated for motility assay. Medium supplemented with 10% FBS was added to the bottom chamber. Cells on the upper surface of filters were wiped out after 24 h, and those on the undersurface were stained with 1% amino toluene blue and counted under a microscope.

Western blotting

To study the effects of *H. pylori* on gastric cancer cells, SGC-7901 cells were cultivated *in vitro* and treated with *H. pylori* (MOI: 100:1). Cell lysate samples were separated on a 12% SDS-PAGE by electrophoresis, and transferred onto nitrocellulose membranes. The blots were blocked for 1 h with 5 % nonfat dry-powdered milk (W/V) in TBS-T buffer and incubated with anti-E-cadherin (1:200). Blots were incubated with peroxidase-conjugated secondary antibodies for 1 h at room temperature. The immunoreactive bands were visualized by enhanced chemiluminescence reagent (Pierce, RockfordIL, USA). The experiment was repeated three times.

Statistical analysis

All the data were given as the mean ± S.D. SPSS 13.0 statistical package (SPSS, Inc., Chicago, IL, USA) was used to analyze the experiment data. Multiple comparisons were done with one-way analysis of variance (ANOVA). Two-group comparisons were performed with a *t*-test. Comparisons of proportions were evaluated by using a chi-square test. P value less than 0.05 was considered statistically significant.

Results

Ultrastructure of H. pylori

After gastric epithelial cells SGC-7901 had been infected with *H. pylori* for 24 h, a number of *H. pylori* were found to surround, attach to, or invade the SGC-7901 cells (Fig. 1A). Based on the appearance under the TEM, *H. pylori* was categorized into three types: (1) a bacillary form with rod or curved shape; (2) a coccoid form with round shape; and (3) an undetermined bacteria form inside the vacuoles, with an irregular outline and inhomogeneous cytoplasm, which we took as dead bacteria. An external membrane can be hardly seen. In the former two forms of *H. pylori*, one showed a homogeneous cytoplasm, whereas the second persistently showed an intracytoplasmic ring-shaped vacuole; both forms had an external membrane.

H. pylori appeared in spiral form with length between 0.8 μm and 2.2 μm, whereas its diameter remained constant around 0.4 μm. The diameter of the coccoid form ranged from 0.2 to 0.5 μm, which was similar to the undetermined bacteria form in size except that the latter was irregular in shape and inhomogenous in electron density (Fig. 1B).

Adhesion and invasiveness of H. pylori to SGC-7901 cells

In this study, we defined adhesion as an attachment of *H. pylori* to the SGC-7901 cells. *H. pylori* were found as either attaching to epithelial cell microvilli or adhering to cell surfaces with adhesion pedestals, or occupying a depression in the cell membrane (Fig. 2A,B). Bacteria observed at intercellular, intracellular, and stromal levels indicated adherence and invasive property.

Ultrastructure of infected cells

The tumor cells SGC-7901 showed polygonal shape, large deformed nuclei, clear nucleoles, and increased chromatin (Fig. 3A). These cells also had well-defined margins. Abundant mitochondrions, some dilated rough endoplasmic reticulum (rER), and free ribosomes were noted in the cytoplasm, and intracytoplasmic microfilaments were also seen (Fig. 3C).

Table 1. Effect of *H. pylori* on SGC-7901 cell cycle (%).

Groups	*H. pylori* (MOI 100:1)		
	G0/G1	S	G2/M
Control group	66.97±3.74	23.26±3.67	9.77±0.39
24 h	45.85±2.26$^{\Delta}$	33.65±0.79$^{\Delta}$	20.5±1.68$^{\Delta}$
48 h	41.05±6.33$^{\Delta}$	54.29±2.78$^{\Delta}$#	4.66±3.55$^{\Delta}$#

The cell cycle of SGC-7901 cells in the presence of *H. pylori* was determined by flow cytometry at 24 h and 48 h; Results presented are representative of 3 independent experimens. $^{\Delta}$: P<0.05 vs control group; $^{\Delta}$#: P<0.05 vs 24 h group

Table 2. Effect of *H. pylori* on SGC-7901 invasion.

H. pylori (100:1)	Cells invaded basement membrane	*P* value
Control group	40.93±4.28	0.000
24 h	79.46±5.19	

After being treated with *H. pylori* (MOI=100:1), the percentage of invasion of SGC-7901 cells was significantly higher in the experiment group (A) than in the control group (B). P=0.000.

There were many changes of cells that were infected with *H. pylori*. The damage was characterized by dilated rER, decreased Golgi, enlarged lysosome and swollen mitochondrions. Lytic cytoplasm lysis and defective cell membrane were outstanding features. However, nuclei and karyotheca were rarely attacked (Figs. 2A,3B,3D). Markedly elongated cells may reflect disruption of intracytoplasmic microfilaments.

Vacuolar degeneration became apparent, which was an important indicator of cellular damage. After being

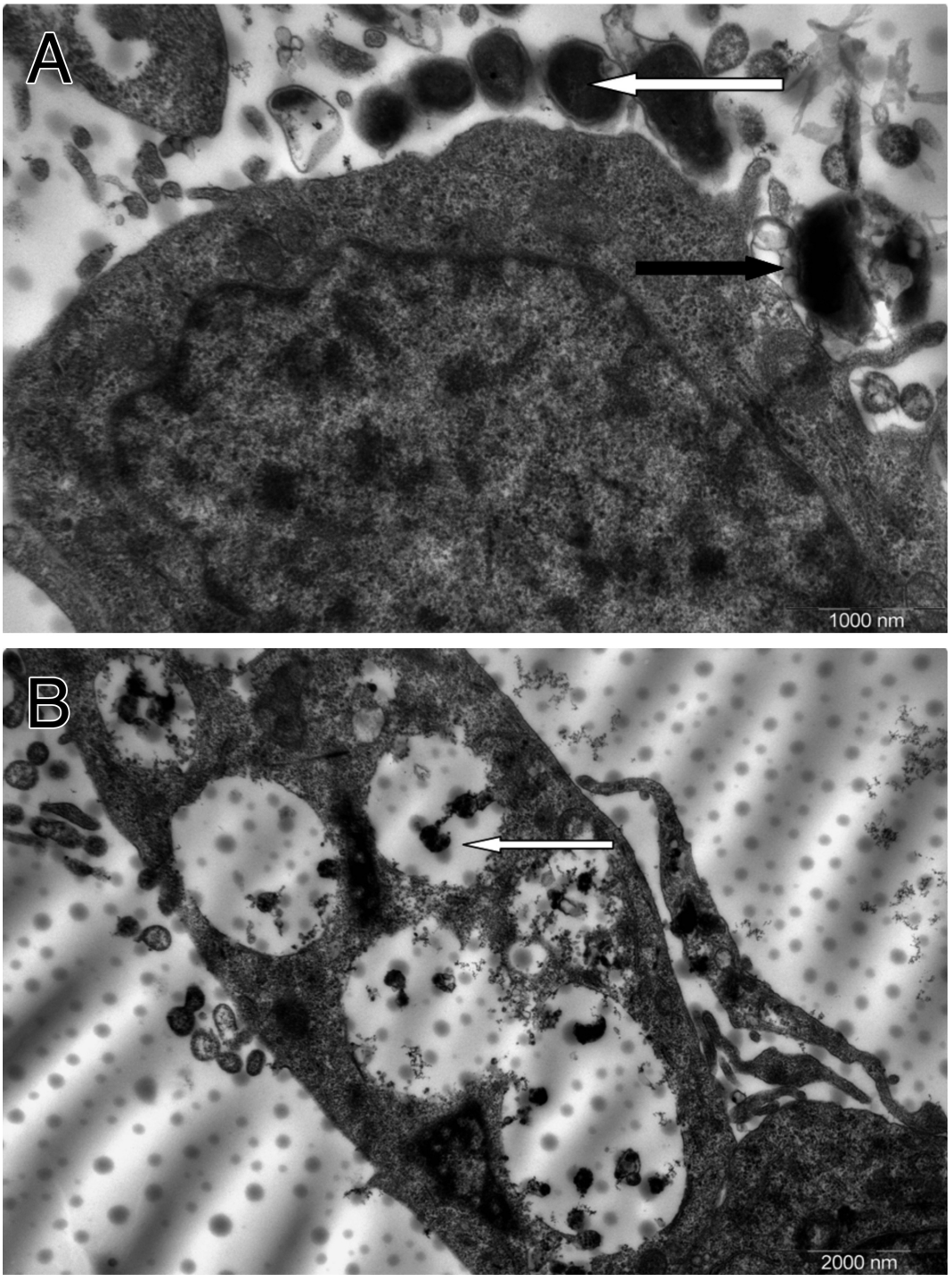

Fig. 1. Interaction between gastric epithelial cells SGC-7901 and *H. pylori*. Gastric epithelial cells SGC-7901 were infected with *H. pylori* strain 26695 for 24 h. Ultrastructure of *H. pylori* was observed by using transmission electron microscopy. Three types of *H. pylori* categorized by morphology shape were exhibited. **A.** The black arrows show rod or cured shapes, the open arrow shows coccoid form. Both of them had an external membrane. **B.** The third type of H. pylor is an undetermined bacteria form inside the vacuoles and was of an irregular outline with inhomogeneous cytoplasm, as well as having no external membrane.

infected with *H. pylori* 6 h, under the light microscope, there were small vacuoles in the cytoplasm around the nucleus. The quantity of vacuoles and dead cells increased as the action time prolonging (Fig. 4).

Effect of H. pylori on cell junction ultrastructure of SGC-7901 cells

Cell junction ultrastructure was observed under

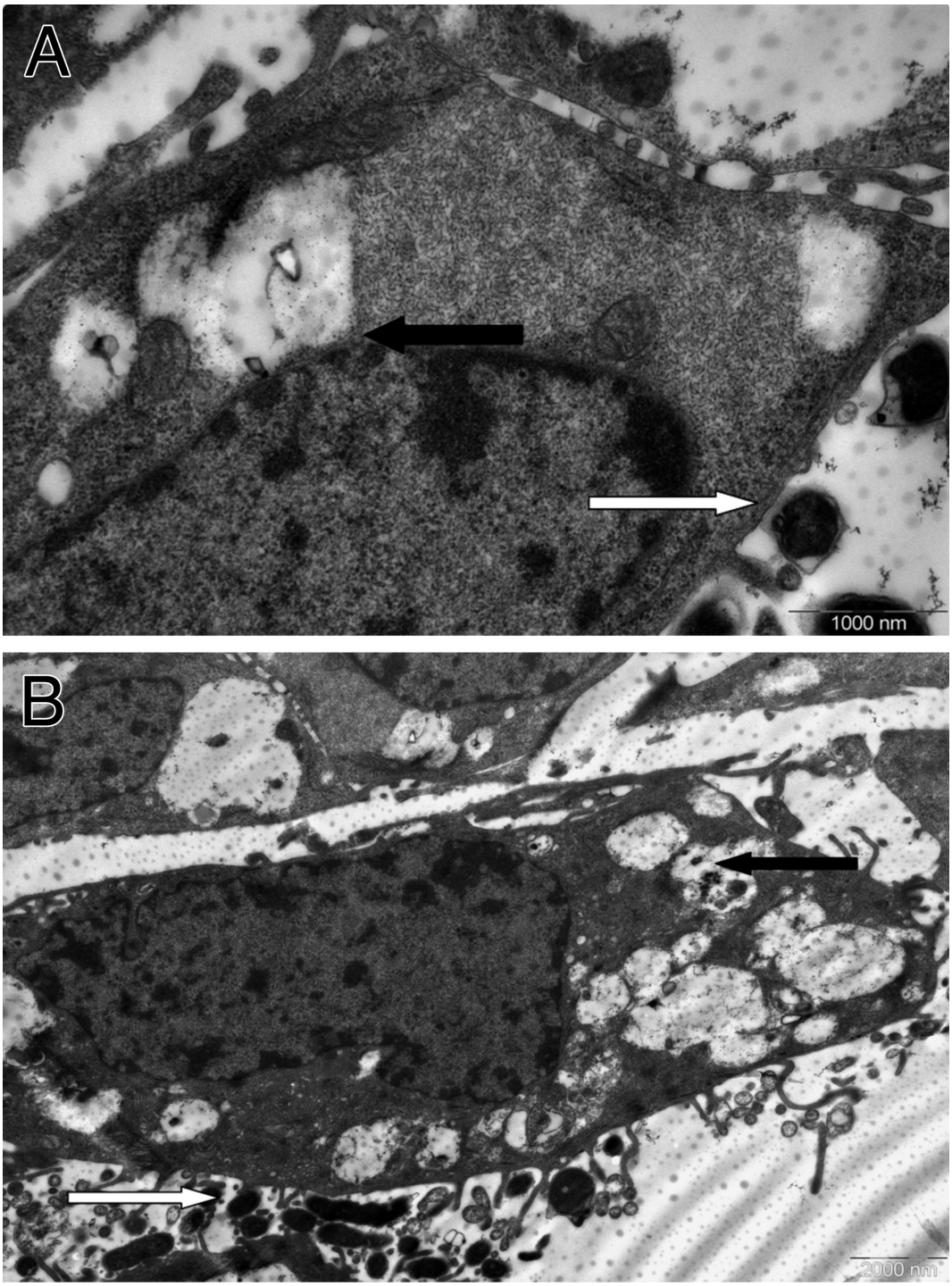

Fig. 2. Adhesion and invasiveness of *H. pylori* to SGC-7901 cells. **A.** *H. pylori* attached to gastric cell surface directly; at the contact areas, membranes of *H. pylori* and gastric epithelium are fused (showed in white arrow). The margin of vacuoles suddenly stopped in karyotheca, whereas nuclear membranes were intact (black arrow). **B.** *H. pylori* adhered to gastric epithelial cell microvilli (white arrow) or invaded cytoplasm (black arrow).

TEM to observe the effect of *H. pylori* on cell junction. After being cultured for 24 h without *H. pylori*, gastric epithelial cells SGC-7901 had distributed more intensively, with long and thin microvilli on the cell surface; the cell gap was smaller, adjacent cell membranes moved closer to each other, and more cell

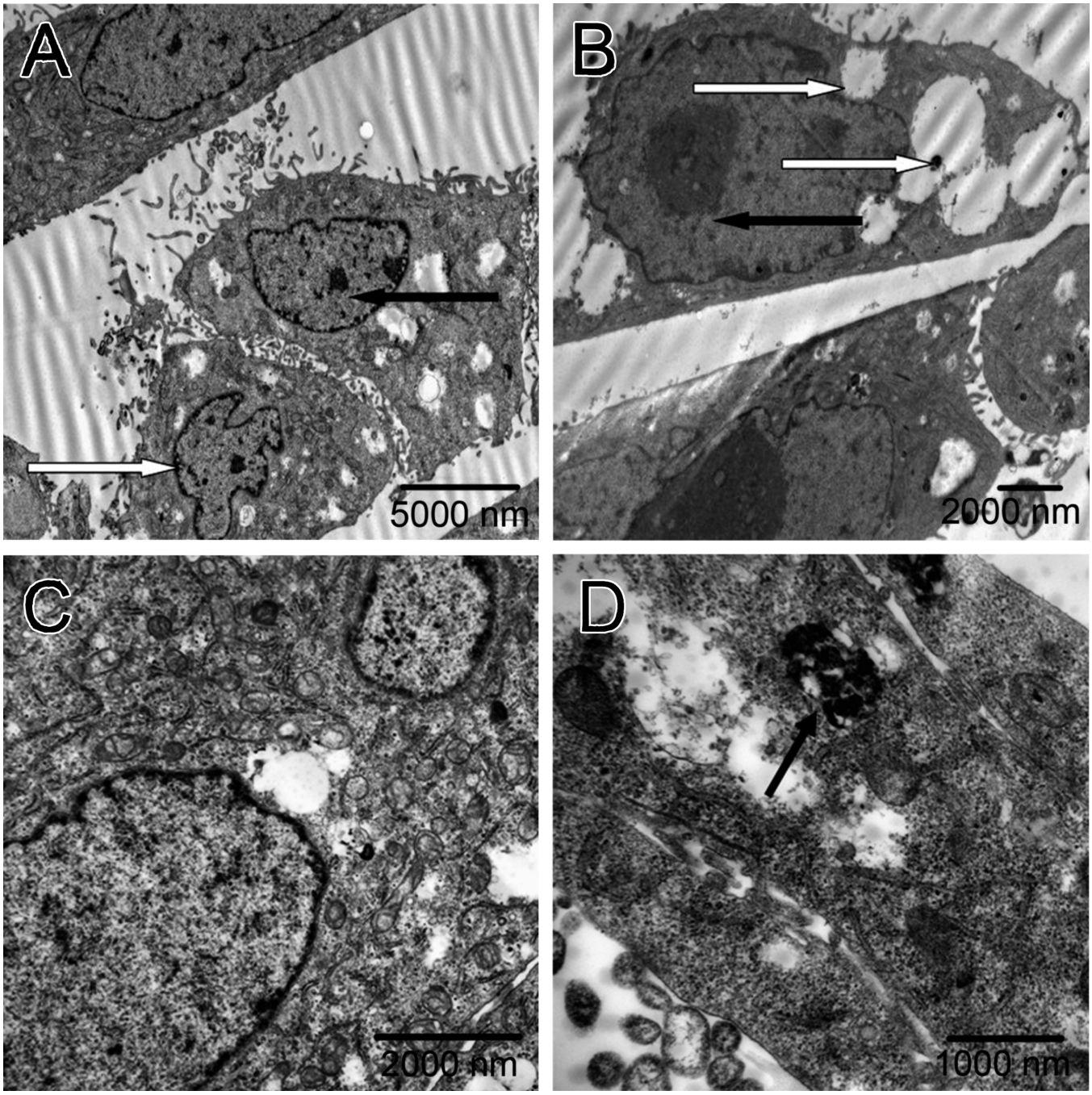

Fig. 3 Ultrastructure of SGC-7901 cells infected by *H. pylori*. Morphologic changes of gastric cancer cells SGC-7901 infected with *H. pylori* under transmission electron microscopy. **A.** The control group cells SGC-7901 showed polygonal shape, large deformed nuclei (white arrow), clear nucleoles (black arrow) and increased chromatin. **B.** The experiment group cells SGC-7901 showed that there were small or large vacuoles in the cytoplasm around the nuclei (white arrow) and increased nucleole (black arrow). **C.** The control group cells showed well-defined margins, abundant mitochondrions, some dilated rER, and the intracytoplasmic myomicrofilaments. **D.** The experiment group cells showed parts of cytoplasmic and organelles lysis and decreased organelles. The arrows point to an enlarged lysosome.

junctions were formed. The junctional complex was noted (Fig.5A). After being cultured for 24 h with *H. pylori*, the distribution of cells was sparser, the intercellular space of cells obviously broadened, and the number of cell contact junctions, including tight junction and desmosome, was reduced or disappeared (Fig. 5B). Meanwhile, microvilli adjacent to *H. pylori* decreased in both length and number, in a time-dependent manner, and they were absent at attachment sites (Fig. 5B).

Apoptosis induced by H. pylori infection

Flow cytometry was used to analyze the apoptosis of SGC-7901 cells co-cultured (infected) with *H. pylori*. After infected with *H. pylori* for 24 h, 48 h, the gastric epithelia did not show increased in apoptotic index at all concrntrtion of MOI=10:1, 100:1, 200. (Fig. 6A,B. shows a representative histogram).

Although there is no apoptotic effect, *H. pylori* has an effect on cell cycle phase distribution (Tab. 1. Fig. 6A,B). When compared with control, *H. pylori* increased the population in the S phase from 23.26 to 33.65% at 24 h and 54.29 at 48 h, consistent with its growth inhibitory effects, and from 9.09 to 32.67% at 24 h and 48 h, with a corresponding decrease in cells in the G1 phase, in a time-dependent manner. This data corroborates the

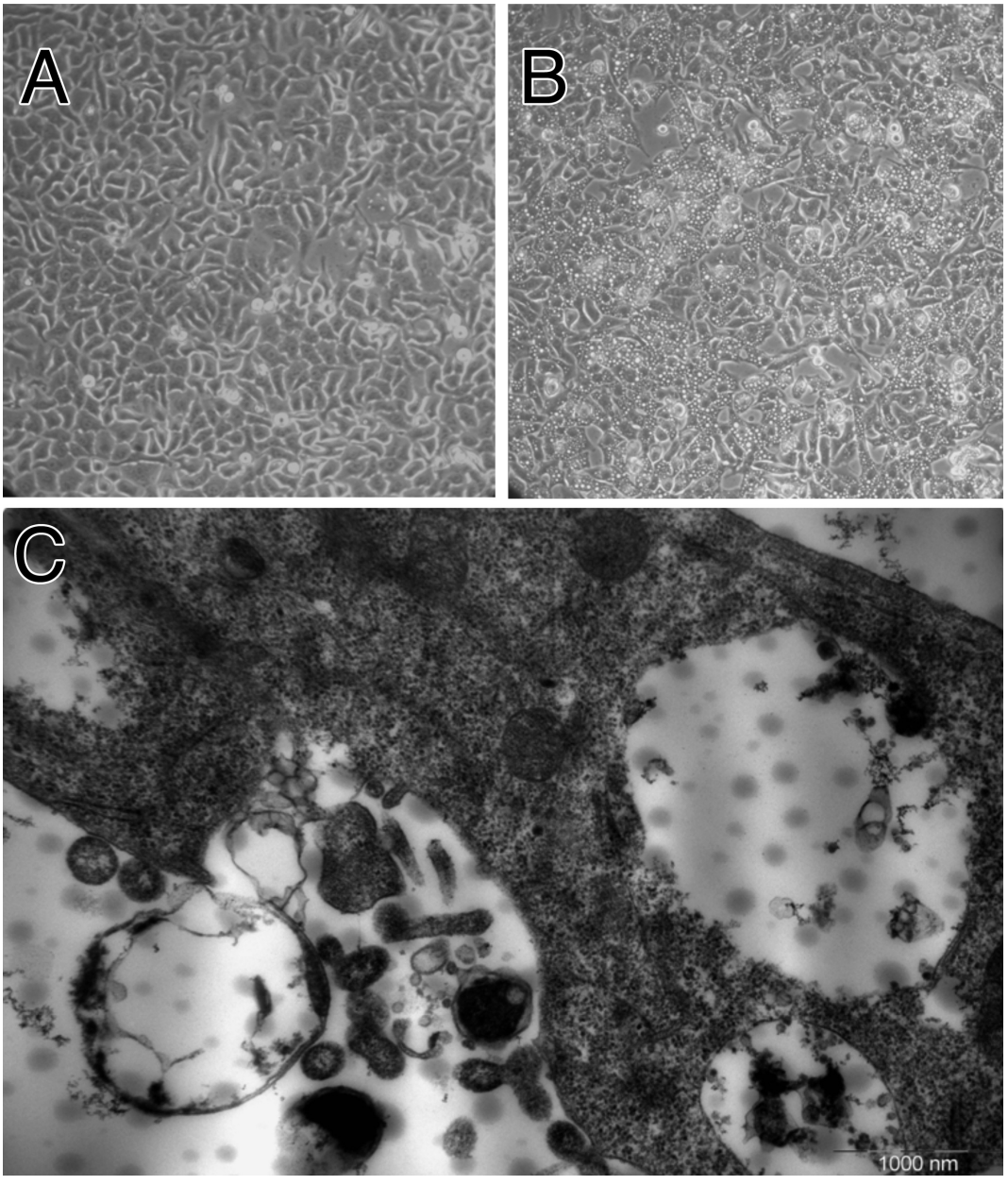

Fig. 4. Morphologic changes of gastric cancer cells SGC-7901 infected with *H. pylori* under the light microscope. **A, B.** left (4A): control group, right (4B): treated group, there were was small vacuoles in the cytoplasm around the nuclei. **C.** The vacuoles under the TEM were irregular in shape. The vacuoles have an obscure margin. *H. pylori*, which is intracellular within cytoplasmic vacuoles, appears as an undetermined form with an irregular outline, inhomogeneous electron density cytoplasm, and lack of an external membrane.

potent inhibitory effect of *H. pylori* on DNA synthesis. Therefor, *H. pylori* inhibites cell proliferation.

Effect of H. pylori on cell proliferation

The effect of H pylori on cell proliferation of SGC7901 cells was evaluated by MTT assay (A 490 nm). Our examination showed that cell viabilty was decreased after co-culture with *H. pylori* (Fig. 7). The inhibition rate was dose and time-dependent.

Effect of H. pylori on SGC-7901 invasion

To further discern the effects of *H. pylori* on invasive properties of gastric adenocarcinoma SGC-7901 cell line, in vitro invasion assays were used. Cells that invaded to the lower surface of the membrane in these assays were fixed and stained. The percentage of invasion of SGC-7901 cells was significantly higher in the *H. pylori* (MOI=100:1) treatment group than in the control group (P=0.000), which indicated that *H. pylori* boosts invasive ability (Fig. 8A,B, Table 2).

Effect of H. pylori on E-cadherin expression in SGC-7901 cells

The effect of *H. pylori* on the protein expression of E-cadherin was examined by Western blot analysis. We detected that the E-cadherin levels decreased in SGC-7901 cells treated with *H. pylori* (MOI=100:1) for 24 h (Fig.9).

Discussion

H. pylori infection is an important etiological risk factor in gastric cancer, and has been classified as a

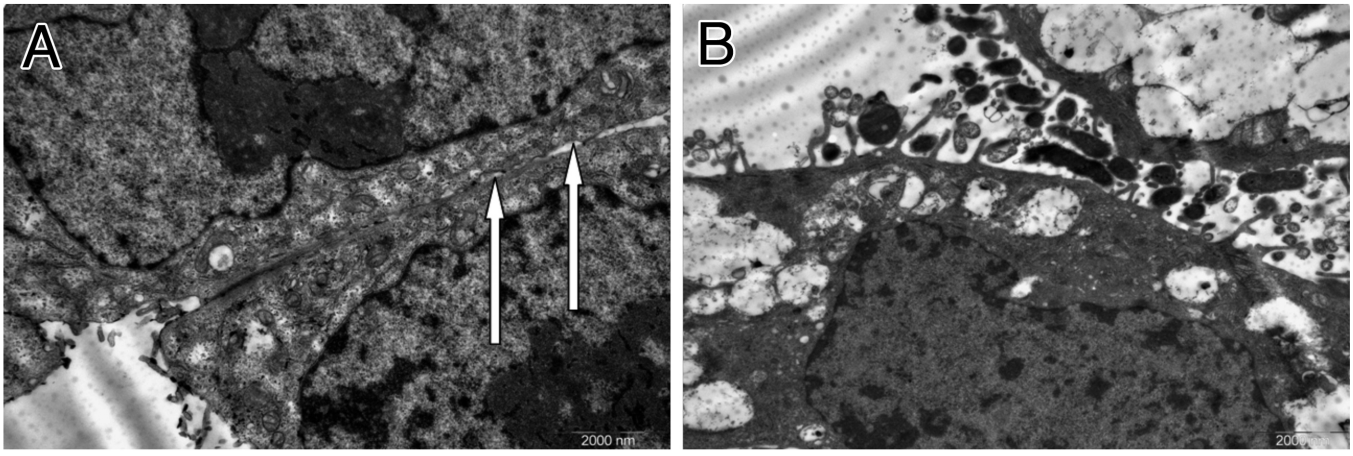

Fig. 5. Effect of *H. pylori* on cell junction ultrastructure of SGC-7901 cells. **A.** After being cultured for 24 h without *H. pylori*, gastric epithelial cells SGC-7901 had distributed more intensively, with long and thin microvilli on the cell surface, the cell gap was smaller, adjacent cell membranes moved closer to each other, and more cell junctions were formed. Black arrow points to a tight junction, whereas white arrow points to desmosomes. **B.** Microvilli adjacent to *H. pylori* decreased in both length and number, and they were often absent at attachment sites. The cell junction of a number of cells was absent with enlarged cell space.

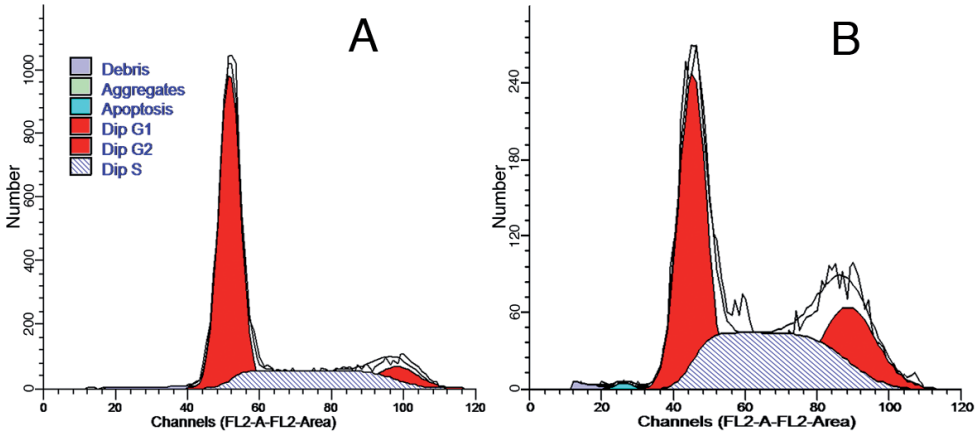

Fig. 6. Effects of *H. pylori* on apoptosis and cell cycle. The apoptosis of SGC-7901 cells induced by *H. pylori* was determined by flow cytometry at 24 h, the graph shows representative flow cytometry histograms of apoptosis and cell cycle. **A.** control group; **B.** treated group.

group I or definite carcinogen by the World Health Organization (World Health Organization, 1994). The mechanisms whereby *H. pylori* exerts its pathogenetic action are not yet well understood. Several morphological studies at electron microscopic level have contributed important information on the relationship between *H. pylori* and gastric mucosa (Caselli et al., 1993; Heczko et al., 2000; Chun et al., 2002; Terebiznik et al., 2006, 2009; Ozbek et al., 2010). Here, we chose international standard *H. pylori* strain 26695, which is $CagPAI^+$ and $VacA^+$ and considered as a high-pathogenicity strain, to study their effects on gastric epithelial cells.

Spiral and coccoid form are often reported in the literature. (Heczko et al., 2000; Chun et al., 2002; Liu et al., 2006). The reason for typed bacteria is that the interaction between cells and *H. pylori* and microenvironment changes. Coccoid formation might occur in response to starvation conditions or when exposed to cells that adapt to the microenvironment (Nilsson et al., 2002).

H. pylori lacking the normal spiral shape has been reported to lose its infectiousness (Cole et al., 1997). Although some investigators have shown that coccoids lose their urease activity, potentially decreasing their virulence, others have demonstrated that coccoids are still able to induce cellular damage (Segal et al., 1996; Andersen et al., 1997). These coccoidal forms were more frequently found in cases of adenocarcinoma than in cases of benign ulcers (Chan et al., 1994). Consistent with that, coccoids here were more easily seen than the typical spiral form after being co-cultured with gastric

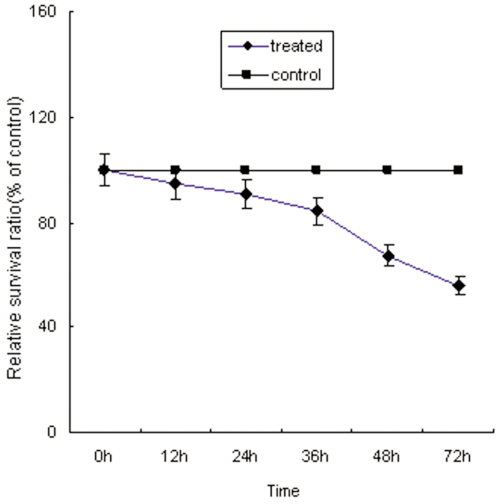

Fig. 7. Effects of *H. pylori* on viability of SGC-7901 cells. Time-dependent inhibition was observed in *H. pylori* treated cells at 0, 12, 24,36, 48, and 72 h. Cell viability was decreased after being co-cultured with *H. pylori*. Data shown are mean ± S.D. by three parallel experiments.

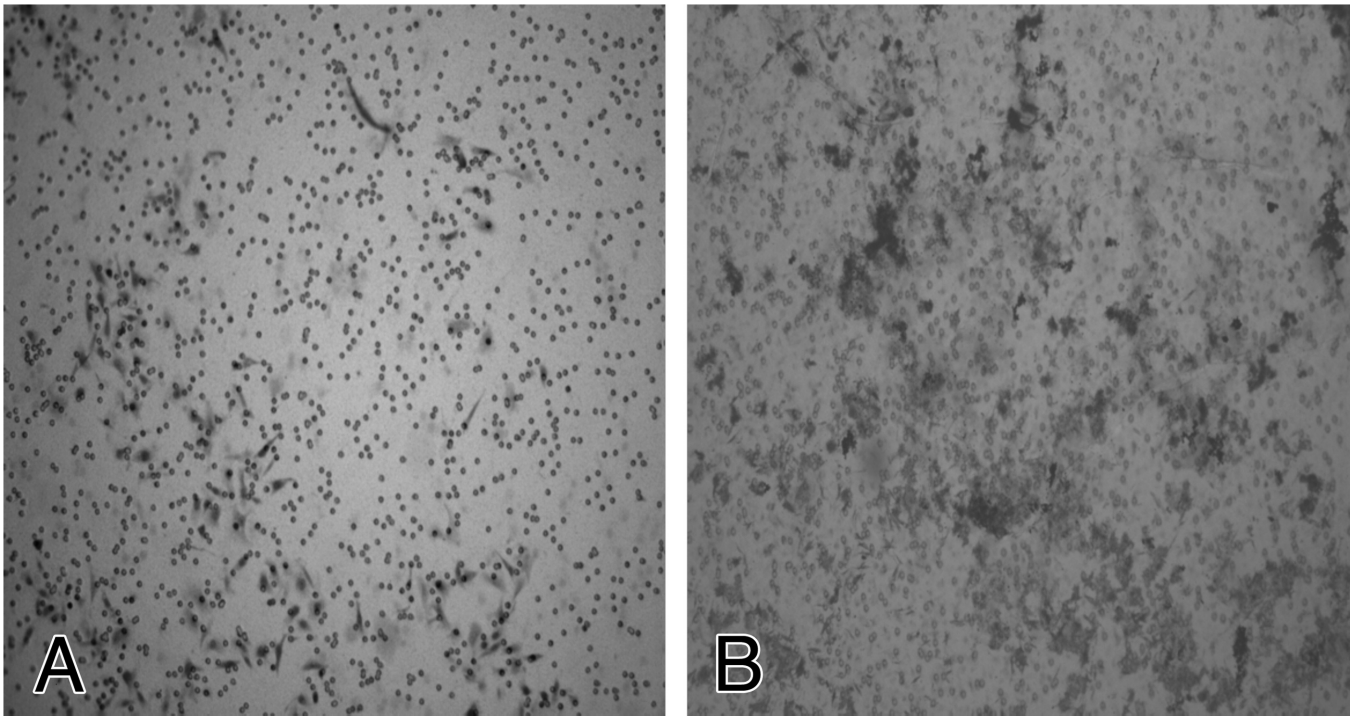

Fig. 8. Effect of *H. pylori* on SGC-7901 invasion. After being treated with *H. pylori* (MOI = 100:1), the percentage of invasion of SGC-7901 cells was significantly higher in the experiment group **(A)** than in the control group **(B).**

carcinoma cells SGC-7901. Coccoids had the same infectious ability as the spiral form in that they can also attach and invade to epithelial cells, and as a result coccoids are still able to induce cellular damage, just as we observed in this study.

Although *H. pylori* is not considered an invasive pathogen, numerous clinical and experimental observations support the notion that *H. pylori* is able to invade epithelial cells in the gastric mucosa. *H. pylori* were also observed inside epithelial cell lines infected with the bacterium (Evans et al., 1992; Björkholm et al., 2000; Amieva et al., 2002; Kwok et al., 2002). It is possible that invasiveness plays a role in *H. pylori* pathogenicity (Petersen and Krogfelt, 2003).

The direct contact between *H. pylori* and epithelial cells seems to be a vital step before invasion and significant cell damage. Dubois group's (Dubois and Borén, 2007; Necchi et al., 2007) observations provide convincing ultrastructural evidence that *H. pylori* can enter and survive within normal and neoplastic epithelial cells. The invasiveness of the bacterium has often been considered relevant for carcinogenesis. Intracellular, intercellular, and stromal invasion of gastric mucosa caused antimicrobial therapy to fail, because epithelial cells instead of *H. pylori* were exposed to antimicrobial drugs.

We report here that a hallmark of cultured epithelial cells with *H. pylori* is the formation of intracellular vacuoles that can occupy a large fraction of the epinuclear region within the cytosol under the transmission electron microscope (Figs. 2B, 3B, 4C), which depends on incubating time and concentration.

The mechanism of cell damage and the process of vacuole formation is not clearly understood. It is well known that *H. pylori* is able to produce large quantities of urease enzyme and other virulence factors. Once *H. pylori* takes residence in or adheres to the epithelial cell, it produces more toxin, and, thus, cell damage occurs. Bacterial virulence factors, such as cytotoxin-associated antigen A (CagA), the cag pathogenicity island (PAI), vacuolating cytotoxin (VacA), urease, and outer membrane proteins (OMPs), are responsible for many of these effects (Cover and Blanke, 2005). This toxin may also contribute to the persistence of *H. pylori* infection by interfering with the MHC class II antigen presentation machinery in antigen-processing cells, potentially leading to suppression of T cell-mediated antigen recognition *in vivo* (Bland et al., 2006).

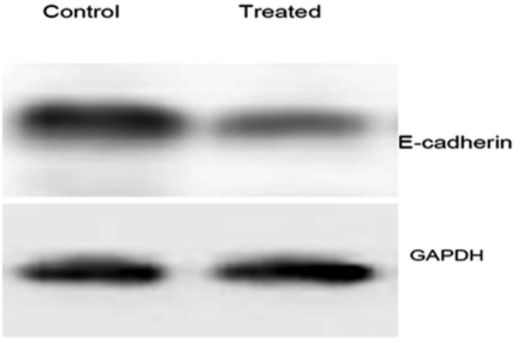

Fig. 9. Effect of *H. pylori* on E-cadherin expression in SGC-7901 cells. The expression of E-cadherin were assessed by Western blotting. Results presented are representative of 3 independent experiments.

There were two phenomena which allowed us to suggest a hypothesis that so-called vacuoles were actually lytic cytoplasm and organelles. One phenomeon was that the shape of the vacuoles were irregular, and membrane-bound compartments were not existent; the other was that some obscure margins of vacuoles was impaired organelles. Obviously, these so-called vacuoles were not the fusion and/or swelling of membrane-bound compartments induced by vacuolating cytotoxin that bacteria secreted.

Previous studies indicate that *H. pylori* was intracellular within cytoplasmic vacuoles where it remained viable (Amieva et al., 2002). Our experiment confirmed that *H. pylori* invaded gastric carcinoma cells and resided inside both large and small vacuoles (Fig. 4C).

Indeed, we believe that *H. pylori* was alive inside cytoplasmic vacuoles after entering epithelial cells at the beginning. As described by Amieva (2002), *H. pylori* entered cytoplasmic vacuoles with viability. However, observation by TEM showed that *H. pylori*, which is intracellular within cytoplasmic vacuoles, appears as an undetermined form with an irregular outline, inhomogeneous electron density cytoplasm, and lack of an external membrane (Fig. 4C). We consider this form of *H. pylori* as dead bacteria, which was induced when they interacted with host cells.

Therefore, we speculate the following process: *H. pylori* actively enters the cells, possessing motile ability and virulence. As time goes by, the toxin secreted by *H. pylori* has damaging effects within the host cells. The toxin erodes the organelles and cytoplasm around itself. Nevertheless, the reaction of host cells, such as increased lysosome approaching extraneous bacteria, also has damaging effects on bacteria. Under the combined effects, irregular lytic areas were formed and became bigger, which we observed under the light microscope as increased vacuoles. Due to the resistance to erosion, karyotheca was hardly impaired. The phenomenon often presented was that the margin of vacuoles had suddenly stopped in karyotheca. The mechanism of vacuolation was apparently different from that proposed by Amieva et al. (2002). Obviously, the vacuoles were not yet autophagic vacuoles related to autophagy, which is a conserved cellular mechanism for the degradation of cellular components in the cytoplasm (Terebiznik et al., 2009). Definitely, they were not the fusion and/or swelling of membrane-bound compartments according to observation under the TEM.

Our data support the notion that *H. pylori* is able to invade epithelial cells, destroy cell structure, and lead to cell death, and the *H. pylori* died as well at same time.

The *H. pylori* was usually seen on the surface or inside the epithelial cells, which was different from that in a construction report by Al-Muhtaseb et al. (2000).

H. pylori might affect the formation of cell gap junction and reduce the gap junction intercellular communication (GJIC) function of the cell, so that the cell loses its contact signal of growth regulation and cell differentiation, thus finally leading to occurrence and progression of gastric cancer. Loss of cell adhesion may account for the ability of cancer cells to cross normal tissue boundaries and metastasize.

The VacA toxin induced apoptosis of gastric epithelial cells (Cho et al., 2003), however, Oldani et al. (2009) demonstrate that CagA blocked VacA-induced apoptosis. By exerting proinflammatory and anti-apoptotic activities, CagA favors, in the long term, the occurrence of the most severe gastric diseases, such as peptic ulcer and gastric cancer (Oldani et al. 2009).

In addition, as recently proposed, the anti-apoptotic activity of CagA might also dampen epithelial gastric cell renewal (Mimuro et al., 2007), whereas VacA decreases CagA-induced cell scattering and motility (Tegtmeyer et al., 2009). From here, we can speculate that the different components of *H. pylori* have different functions, and there are kinds of cross-talks among them. Studies on only some parts of the *H. pylori* component might not achieve comprehensive results as a whole.

Except for the reasons mentioned above, we believe, the reason for the antiapoptosis of *H. pylori* is that they have too many toxic effects and cause degeneration and necrosis instead of apoptosis.

Several studies reported that *H. pylori* can inhibit cell proliferation in vitro (Smoot et al., 1999; Pearce et al., 2004), which is consistent with the results of this study. One reason for the antiproliferation, we speculate, is that *H. pylori* has toxic effects on gastric epithelial cells directly. Another is probably related to the reduction of cell contacts. Loss of cell contacts impedes cell signal transduction, therefore inhibiting cell proliferation. Deceasing cell proliferation may increase the chances of ulcer formation and delay ulcer healing, which seem to be relevant to the pathogenesis of *H. pylori*-associated peptic ulcer diseases.

Our results clearly demonstrate that *H. pylori* effectively promotes cancer cell invasion *in vitro*. These data suggest that *H. pylori* may play a key role in the acquisition of the ability to invade basement membrane barriers.

Metastasis remains a major cause of poor prognosis and death in patients with cancer even after curative resection. The process of metastasis consists of sequential steps that include detachment, motility, invasion of the extracellular matrix, intravasation, circulation, adhesion, extravasation into the organ parenchyma, and growth.

The detachment of cancer cells from their parent tumors is an early initial event in metastasis. Many factors affect cell detachment: growth rate, necrosis, enzyme activity, and stress on cell release. A loss of cell-cell adhesive interaction is required for detachment. Thus, adhesion molecules play an important role in the metastatic process. E-cadherin is essential to the maintenance of cell morphology, cell movement and cell adhesive function. Several studies demonstrated that reduction or structural alternation of E-cadherin expression promotes cancer metastasis. In this study, we detected that *H. pylori* decreased E-cadherin expression (Fig. 9), which facilitated cell invasion and resulted in metastasis.

This study showed that *H. pylori* affects the cell junction, results in wider intercellular space, loss of cell contacts, and reduction of cell microvilli.

Detachment of gastric epithelial cells may decrease the cell GJIC function, increase cell mobile ability, and promote cell invasiveness.

The degradation of the extracellular matrix is another step in metastasis. Krueger et al., 2006 reported that *H. pylori* induced overexpression of MMP-1, resulting in tissue architecture loosening and degrading reconstituted basement membrane, therefore facilitating invasiveness and metasitasis.

If we can early eradicate *H. pylori* infection at the promotion stages in gastric carcinogenesis, and recover the GJIC function, then we may have a good chance to stop the further development of gastric cancer.

Conclusions

In summary, *H. pylori* damages cell construction, destroys cell junctions, inhibits cell proliferation, promotes cell invasive ability, and, therefore, might accelerate the malignant progress of gastric cancer. The exact molecular mechanism and in vivo studies are necessary to further confirm our findings.

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