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ORIGINAL ARTICLE

Basic Study

Impact of *Helicobacter pylori* on the healing process of the gastric barrier

Eliza Mnich, Magdalena Kowalewicz-Kulbat, Paulina Sicińska, Krzysztof Hinc, Michał Obuchowski, Adrian Gajewski, Anthony P Moran, Magdalena Chmiela

Eliza Mnich, Magdalena Kowalewicz-Kulbat, Adrian Gajewski, Magdalena Chmiela, Department of Immunology and Infectious Biology, Institute of Microbiology, Biotechnology and Immunology, Faculty of Biology and Environmental Protection, University of Łódź, 90-237 Łódź, Poland

Paulina Sicińska, Department of Biophysics of Environment Pollution, Faculty of Biology and Environmental Protection, University of Łódź, 90-237 Łódź, Poland

Krzysztof Hinc, Michał Obuchowski, Laboratory of Molecular Bacteriology, Intercollegiate Faculty of Biotechnology UG-MUG, Medical University of Gdańsk, 80-210 Gdańsk, Poland

Anthony P Moran, Department of Microbiology, Laboratory of Molecular Biochemistry, University College, 9014 Galway, Ireland

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Correspondence to: Magdalena Chmiela, Professor, PhD, Department of Immunology and Infectious Biology, Institute of Microbiology, Biotechnology and Immunology, Faculty of Biology and Environmental Protection, University of Łódź, Banacha 12/16, 90-237 Łódź, Poland. chmiela@biol.uni.lodz.pl
Telephone: +48-42-6354186
Fax: +48-42-6655818

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Abstract

AIM

To determine the impact of selected well defined *Helicobacter pylori* (*H. pylori*) antigens on gastric barrier cell turnover.

METHODS

In this study, using two cellular models of gastric epithelial cells and fibroblasts, we have focused on exploring the effects of well defined *H. pylori* soluble components such as glycine acid extract antigenic complex (GE), subunit A of urease (UreA), cytotoxin associated gene A protein (CagA) and lipopolysaccharide (LPS) on cell turnover by comparing the wound healing capacity of the cells in terms of their

proliferative and metabolic activity as well as cell cycle distribution. Toxic effects of *H. pylori* components have been assessed in an association with damage to cell nuclei and inhibition of signal transducer and activator of transcription 3 (STAT3) phosphorylation.

RESULTS

We showed that *H. pylori* GE, CagA and UreA promoted regeneration of epithelial cells and fibroblasts, which is necessary for effective tissue healing. However, *in vivo* increased proliferative activity of these cells may constitute an increased risk of gastric neoplasia. In contrast, *H. pylori* LPS showed a dose-dependent influence on the process of wound healing. At a low concentration (1 ng/mL) *H. pylori* LPS accelerated of healing epithelial cells, which was linked to significantly enhanced cell proliferation and MTT reduction as well as lack of alterations in cell cycle and downregulation of epidermal growth factor (EGF) production as well as cell nuclei destruction. By comparison, *H. pylori* LPS at a high concentration (25 ng/mL) inhibited the process of wound repair, which was related to diminished proliferative activity of the cells, cell cycle arrest, destruction of cell nuclei and downregulation of the EGF/STAT3 signalling pathway.

CONCLUSION

In vivo H. pylori LPS driven effects might lead to the maintenance of chronic inflammatory response and pathological disorders on the level of the gastric mucosal barrier.

Key words: *Helicobacter pylori*; Wound healing; Gastric barrier dysfunction

Core tip: This manuscript focused on the impact of *Helicobacter pylori* (*H. pylori*) antigens to the gastric mucosal barrier. We evaluated the effects of *H. pylori* antigens using *in vitro* two cellular models of gastric epithelial cells and fibroblasts, which had been independently exposed to *H. pylori* components. In this study, we showed different effects of subunit A of urease, cytotoxin associated gene A protein, lipopolysaccharide (LPS) as well as compounds included in a glycine acid extract on the regenerative activity of gastric epithelial cells and fibroblasts. Our results indicate deleterious, dose dependent influence of *H. pylori* LPS on this process.

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INTRODUCTION

The gastric mucosal barrier (GMB) is composed of a pre-epithelial layer (mucus and bicarbonate), a tight epithelial component, the post-epithelial layer (fibroblasts and immune cells), microcirculation (blood flow) and nerves[1]. Epithelial cells are responsible for gastric barrier integrity and function[2]. Any disruption of GMB, due to infectious agents or inflammation, leads to a variety of disorders, including gastritis or even gastric cancer. In order to establish and develop a disease, infectious agents must overcome GMB[3]. Among bacterial pathogens, a Gram-negative, spiral-shaped bacterium *Helicobacter pylori* (*H. pylori*) has been shown to play a crucial role in the development of gastritis and gastric as well as duodenal ulcers[4] due to various mechanisms to evade host's responses[5]. In 10%-15% individuals this infection can lead to severe inflammation, peptic ulcer disease (10%), mucosa-associated lymphoid tissue (MALT) lymphoma (0.1%), or gastric adenocarcinoma (1%-3%)[6-9]. *H. pylori* induces histological gastritis associated with an infiltration of gastric mucosa with immune cells[10]. However, other microorganisms or even non-infectious agents such as corticosteroids, nonsteroidal anti-inflammatory drugs, aspirin and excessive alcohol consumption can play a role in the development of gastritis[11-13]. *H. pylori* antigens, which are translocated through the gastrointestinal tract in the Payer's patches, induce specific immune response[14]. Small molecular weight antigens including LPS enter the lamina propria *via* goblet cells. Moreover, the epithelial cells villi can also internalize particles of antigens such as bacterial cell debris, which can be found co-localized with $CD11^+$ dendritic cells in the lamina propria[15].

The infection begins by mucus colonization, which is followed by the attachment of bacteria to the underlying epithelial cells and extracellular matrix proteins[16-18]. The bacteria also interact with infiltrating immune cells *via* Pathogen Recognition Receptors (PRR) stimulating them to cytokine secretion or can even enter the bloodstream[19,20]. *H. pylori* urease protects the pathogens from gastric acid and degrades of intracellular tight junctions[21-23]. Adhesins representing outer membrane proteins such as Hop proteins and blood antigen binding adhesins mediate *H. pylori* binding to GMB[16,18]. Other factors, such as cytotoxin-associated gene A (CagA) protein and vacuolating toxin A (VacA) are able to trigger inflammatory responses in host gastric tissues and predispose to gastric ulcer and cancer[6,24]. The CagA is delivered into the host cells by the type IV secretion system (T4SS)[25-27] where it interferes with host signalling pathways and cellular functions[28,29]. However, CagA may also interact with the host cells in a soluble form[30,31] or as phospholipid vesicles[32,33], which have been indentified to attach to and to be taken up by

human epithelial cells[34-36]. Furthermore, it has been found that gastric epithelial cells inducibly expressing CagA secrete exosomes containing CagA, which can be distributed by circulation[37]. By using the *H. pylori* G27 strain (*cagA*+/*vacA*+) and two isogenic mutants defective in *cagA* (G27 *cagA*-/*vacA*+) or *vacA* (G27 *cagA*+/*vacA*-)[38], we showed that CagA present in the cytoplasmic fraction of bacterial cells was responsible for the inhibition of proliferation of T lymphocytes[28].

Among *H. pylori* virulence factors, LPS has a unique status since modifications of lipid A lead to reduction of endotoxic properties, whereas O-specific chains structurally similar to human Lewis (Le) blood-group antigens are responsible for molecular mimicry[39,40], which allow *H. pylori* to persist[41-43]. This is by reducing the host immune response mechanisms including phagocytosis[44], Natural Killer cells activity[45] and proliferation of T lymphocytes[46-48]. LPS through binding with dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) may interfere with the development of specific immune response[49,50]. The biological actions of LPS are mediated by CD14 and Toll-like receptors (TLR) 4 and TLR2, scavenger receptors, β2 integrins and LPS-binding protein (LBP)[51,52].

The long-term inflammation can increase the gastric barrier permeability as well as further damage to lamina propria[5,50] and might promote different extragastric disorders[53-55]. Although several *H. pylori* factors engaged in gastric lining disruption have been identified, the mechanisms of tissue damage are still not well known. We hypothesised that gastric epithelial barrier disruption could result in either epithelial cell loss due to ulceration or excessive epithelial cell growth predisposing to gastric neoplasia. The aim of this study was to explore the ability of gastric epithelial cells and fibroblasts to heal wound after the challenge with selected *H. pylori* antigens: glycine acid extract antigenic complex (GE), subunit A of urease (UreA), CagA and LPS. We used *in vitro* cellular models to assess the effectiveness of the cells in the wound healing by monitoring the cell migration in association with cell metabolic activity, proliferation, cell cycle distribution, as well as damage to cell nuclei.

MATERIALS AND METHODS

Cell culture

The human AGS (CRL-1739) gastric adenocarcinoma epithelial cell line[56] and guinea pig fibroblasts (CRL-1405)[57] were obtained from the American Type Culture Collection (ATCC, Rockville, Md.). The cells were routinely grown as a monolayer in complete RPMI-1640 medium (cRPMI; Sigma St. Louis, MI, United States), containing 10% heat inactivated Fetal Bovine Serum (FBS; CytoGen, Łódź, Poland), 1% penicillin/streptomycin (Gibco, Zug, Switzerland), at 37 ℃ in a humidified atmosphere containing 5% CO_2. The cells were passaged every seven days with 0.25% trypsin/0.02% EDTA (HyClone, Thermo Fisher Scientific, Waltham, MA, United States) and the medium was changed every 3-4 d.

Stimuli

GE from the reference *H. pylori* strain CCUG 17874 (Culture Collection University of Gothenburg, Sweden), at 10 μg/mL was used in the experiments. Surface *H. pylori* antigens were extracted using 0.2 mol/L glycine buffer, pH 2.2, as previously described[58,59] with the evaluation of protein composition by SDS-PAGE electrophoresis and Western blot - Immuno blot (Milenia® Blot *H. pylori*, DPC Biermann, GmbH, Bad Nauheim, Germany). Serological detection of antigens was performed with reference serum samples from patients infected with *H. pylori*[60]. Major proteins in GE recognized by sera from *H. pylori* infected patients were: 120 kDa (CagA), 87 kDa (VacA), 66kDa (UreB), 60 kDa (Hsp), 29 kDa (UreA), between 66-22 kDa. The protein concentration in GE was 600 μg/mL (NanoDrop 2000c Spectrophotometer, ThermoScientific, Wlatman, WY, United States). GE contained < 0.001 EU/mL of LPS, as shown by the chromogenic *Limulus amebocyte* lysate test (Lonza, Braine-Alleud, Belgium).

Recombinant CagA protein - rCagA (a kind gift from Antonello Covacci, IRIS, Siena, Italy) was used at the concentration of 1 μg/mL. A recombinant fragment of the CagA antigen of *H. pylori*: nt 2777 to nt 3465 of *cagA* gene was used. It was expressed (QIAexpress System, Qiagen, Hilden, Germany) in *E. coli* as a fusion protein (about 26 kDa size) with a 6 His-tail in front of a 230 aa polypeptide of CagA. The protein was purified by Ni^{2+}-NTA agarose column[61] and checked for serological activity in the enzyme immunoassay[62].

Based on the common substrate activity and high homology of urease produced by the species of the genus *Helicobacter* in this study the UreA subunit from *H. acinonychis* isolated from the acidic environment of cheetah stomach was used as a homologue of *H. pylori* UreA protein (97% homology). The urease gene was amplified by a polymerase chain reaction (PCR), as previously described[63] using chromosomal DNA as a template and oligonucleotides hisureA-up and hisureA-dn as primers. DNA encoding six histidines (His6-tag) was carried by oligonucleotide hisure-A-dn. The obtained PCR product of 737 bp was digested with enzymes *KpnI* and *NheI* and cloned into the commercial vector pBAD (Stratagene, California, United States). The resulting plasmid, pMD1, was verified by restriction analysis and nucleotide sequencing. pMD1 was used to transform the *E. coli* strain DH5α and the recombinant strain was used to overproduce UreA by the addition of arabinose 0.05%. A 27 kDa protein was visualized on a coomassie blue stained gel and purified on Ni-NTA superflow agarose (Qiagen) followed by gel filtration on Superose 6 resin. UreA was used at 5 μg/mL.

LPS from the reference strain of *H. pylori* CCUG

17874 was prepared by hot phenol-water extraction as previously described[64]. Whole cell lysates were pretreated with proteinase K (Sigma, St Louis, MI, United States)[65]. Crude extraction of LPS from bacteria was performed with 45% aqueous phenol at 68 ℃ for 30 min[66]. The LPS preparations were purified by treatment with RNase A, DNase II and proteinase K (Sigma) and by ultracentrifugation at 100000 × *g* at 4 ℃ for 18 h[67]. *H. pylori* LPS was used at two concentrations: 1 ng/mL and 25 ng/mL. Standard *E. coli* LPS was used at 1 ng/mL or 25 ng/mL, as a control of *H. pylori* LPS (serotype O55: B5; Sigma).

In order to study the synergistic or antagonistic effects, the following combinations of antigens were used: (1) *H. pylori* LPS (25 ng/mL) + CagA (1 μg/mL); and (2) *H. pylori* LPS (25 ng/mL) + CagA (1 μg/mL) + UreA (5 μg/mL) + GE (10 μg/mL). The antigen concentrations were adjusted experimentally or adopted from previously performed experiments[44,47,48,68].

Scratch wound assay

Cell migration was evaluated based on the ability of the cells to migrate into an empty space created by an *in vitro* scratch wound as previously described[69]. AGS cells or guinea pig fibroblasts were seeded in six-well plates at the density of 1 × 10^6 cells/well in 1 mL/well RPMI-1640 medium supplemented with 2% FBS/1% standard antibiotics and cultured until reaching 100% confluence. A lower percentage of FBS was used to minimize cell proliferation, and sufficient to prevent apoptosis and/or cell detachment. The cell monolayer in each well was physically disrupted with a sterile 200 μL pipette tip, and designated as time 0 h of wound repair. The stimuli described above were added to the cells. The control for a migratory assay consisted of untreated cells alone, which exhibited the normal capacity to migtate. Twenty-four hours after the challenge, antigens were removed from the cell cultures and the cells were washed twice with culture medium/2%FBS. Wound images were taken at 0, 24, 48 and 72 h by a digital camera (Nikon P20, Tokyo, Japan) at the same positions. Areas were measured using the software ImageJ version 1.48v (National Institute of Health, United States). Each wound was measured four times and the average value was assessed. The wound healing in the milieu of antigens was expressed as a percentage of cells migrating to the wound zone in comparison to untreated cells.

Cell viability assay

Cytotoxic effects of stimulators used in this study were evaluated using a tetrazolium yellow dye MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], which is reduced by living cells to yield soluble purple formazan crystals that can be detected colorimetrically. AGS cells and guinea pig fibroblasts (1 × 10^6 cells/well) were placed in 96-well plates in a volume of 100 μL and left to adhere overnight. Subsequently, all cells were washed with cRPMI and incubated for further 24 h with the described stimuli. Fresh MTT solution (5 mg/mL in sterile PBS; Sigma St. Louis, MI, United States) was added to each well and the plates were incubated for 2 h at 37 ℃, 5% CO_2. Formazan crystals were dissolved with acidic isopropanol (0.1 mol/L HCl in absolute isopropanol). Absorbance at 570 nm was estimated with a plate reader Victor2 (Wallac, Oy, Turku, Finland). All results were presented as the percentage means ± SD (standard deviation) relative to untreated cells of at least four independent experiments performed in triplicates. The effectiveness of MTT reduction was calculated based on the following formula: MTT reduction relative to untreated cells (%) = (absorbance of treated cells/absorbance of untreated cells × 100%) - 100%.

Cell proliferation study

A radioactive proliferation assay based on the measurement of the tritiated thymidine ([^{3}H]TdR) incorporation during DNA synthesis was used for the quantification of AGS cells and guinea pig fibroblasts proliferation. These cells were seeded at a density of 1 × 10^6 cells/well into 96-well microplates in 100 μL/well of cRPMI medium and preincubated overnight in order to obtain a monolayer of adherent cells. Further, the cells were stimulated for 24 h in the presence of bacterial antigens or in culture medium alone (as a control of spontaneous proliferation). After stimulation, the antigens were removed by washing the cells with cRPMI. In all experiments, wells containing the cells alone (without any antigens) were included as a control. At 18 h before the end of cultivation, 1 μCi of [^{3}H]TdR (Lacomed, Prague, Czech Republic) was added to each well to estimate cell proliferation. The incorporation of thymidine was measured using a MicroBeta 2 scintillation counter (Wallac Oy, Turku, Finland) after harvesting the cells on fibre filters. All cultures were settled in six repeats. The results were expressed as mean counts per minute (cpm)/culture ± SD of six independent experiments, performed in triplicates. The stimulation index (SI), expressing the relative cpm ratio, was calculated by dividing the counts/min for the cell cultures with a stimulator by the cpm counts/min for the cell cultures without a stimulator. SI values higher than or equal to 1.0 (*cut-off*) were considered as a positive result in the proliferation assay.

Cell cycle analysis

The cell cycle was assessed as previously described[70,71]. Briefly, AGS cells or guinea pig fibroblasts (1 × 10^6 cells/mL) were seeded in 6-well plates (NUNC, Denmark) in 1 mL/well of cRPMI. After overnight preincubation, the cells were cultured for 24 h in the presence of bacterial stimuli or in culture medium alone (as a control). After stimulation, the cells were harvested by trypsinization

and fixed in 70% ice-cold ethanol. The cells were stained for the total DNA content with a solution containing 75 μmol/dm^3 propidium iodide (PI) and 50 IU Kunitz/mL of DNase-free RNase (Sigma St. Louis, MI, United States) in PBS for 30 min, at 37 ℃. The cell cycle distribution was then analyzed in an LSR II Flow Cytometer (Becton Dickinson, Mountain View, CA, United States). The percentage of cells in G_1, S and G_2/M phases of the cell cycle, and the percentage of cells undergoing apoptosis were determined with the FlowJo analytical software.

DAPI staining of cell nuclei

Cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; Sigma, St. Louis, MI, United States), a fluorescent dye which has a strong affinity to the AT base pair in DNA. The cells, after a 24 h stimulation with bacterial antigens, were fixed with 4% formaldehyde, and stained with DAPI solution (2.5 μg/mL) for 15 min at room temperature. Preparations were viewed under a fluorescent microscope (Zeiss, Axio Scope, A1) at a wavelength of 358 nm (excitation) and 461 nm (emission). We evaluated the percentage of the cells with damaged nuclei.

Comet assay

The comet assay was used to detect DNA damage. It was performed under alkaline conditions (pH > 13) as previously described[72]. Briefly, AGS and fibroblast cell suspensions were separately mixed with low-melting point agarose at 1 × 10^4 cells/mL, at 37 ℃ and evenly pipetted onto the microscope slides pre-coated with 250 μL of 0.5% normal melting point agarose. The slides were maintained on ice for 10 min to solidify. All the steps were conducted in the dark or under reduced light to prevent additional DNA damage. The remaining cells were exposed to bacterial stimuli for 24 h. After incubation, the treated cells were washed with ice-cold PBS and spread on the slides as described above. The slides were then immersed in a chilled lysis solution (2.5 mol/L NaCl, 100 mmol/L EDTA, 10 mmol/L Tris-HCl, 1% Triton X-100 and 1% N-lauroylsarcosine sodium, pH 10.0) for 1 h at 4 ℃ in the dark. Thereafter, the slides were rinsed in freshly prepared and chilled electrophoresis buffer (1 mmol/L EDTA, 300 mmol/L NaOH and pH > 13) at 4 ℃ for 40 min to allow DNA unwinding. Electrophoresis was then performed at 25 V, 300 mA (0.86 V/cm) for 23 min at 4 ℃. The slides were washed with a neutralizing buffer (0.4 mol/L Tris-HCl and pH 7.5) and then DNA was stained with DAPI (2 μg/mL). Images of the comets were captured under a fluorescence microscope (Zeiss, Axio Scope. A1) at × 400 magnification. For each sample, a minimum of 100 comets were randomly selected and the percentage of DNA in the comet tail (% tail DNA) was analyzed using the Comet Assay Software Project (CASP) as recommended by Końca *et al*[73].

Apoptosis detection assay

The binding of annexin V-fluorescein isothiocyanate (Ann-V) to externalized phosphatidylserine was used as a marker of apoptotic AGS cells and fibroblasts detected by flow cytometry as previously described[70,71]. Cells were seeded at a density of 1 × 10^6 cells/well into 6-well microplates (NUNC, Denmark) in 1 mL/well of cRPMI. After overnight preincubation, the cells were cultured for a further 24 h in the presence of bacterial stimuli or in culture medium alone (as a control). The fluorescein isothiocyanate (FITC) Annexin V Apoptosis Detection Kit (Becton Dickinson, San Jose, CA, United States) was used for the differentiation of apoptotic and necrotic cells. Briefly, after stimulation, the cells were harvested by gentle trypsynization and washed with cold PBS. The cells were resuspended in 1 mL of 1 × binding buffer. Next, 100 μL was transferred to a 5 mL flow cytometry tube, and incubated with 5 μL of Annexin V and 5 μL of PI for 15 min, at room temperature in the dark. Next, 400 μL of 1 × binding buffer was added to each tube. Flow cytometric analysis was performed immediately after staining.

Annexin V/PI fluorescence was analysed for each sample; 10000 events were collected and fluorescence was detected using FlowJo software. The results are presented as percentages of cells that were viable (Ann-V$^-$/PI$^-$), early apoptotic cells (Ann-V$^+$/PI$^-$), the cells in the late stages of apoptosis (Ann-V$^+$/PI$^+$) or necrotic cells (Ann-V$^-$/PI$^+$).

ELISA assays

The epidermal growth factor (EGF) concentration was evaluated in supernatants from AGS cell cultures untreated or stimulated for 24 h with bacterial antigens, according to the manufacturer's protocol (Human EGF ELISA Kit, Elabscience Biotechnology Co., Ltd, China). Since there is a positive correlation between the concentration of EGF and the signal transducer and activator of transcription 3 (STAT3) signalling pathway, we also assessed the percentage of phospho-STAT3. The procedure detecting phospho-STAT3 in AGS cells was used according to the manufacturer's instructions (Human/Mouse phospho-STAT3 (Y705) Cell-Based ELISA, R&D Systems, Minneapolis, United States). Briefly, 100 μL of 2 × 10^4 AGS cells was seeded into each well of a black 96-well microplate with a clear bottom, and incubated overnight at 37 ℃. The cells were then treated with bacterial antigens for 24 h as previously described. AGS cells stimulated with EGF (0.125 ng/mL) were used as a positive control. Following the treatments, the cells were tested with the cell-based ELISA kit.

Statistical analysis

All values were expressed as the mean ± SD. The differences between antigen activities were tested using the non-parametric Mann-Whitney *U* test. For

statistical analysis the Statistica 12 PL software was used. Results were considered statistically significant when $P < 0.05$.

RESULTS

Kinetics of wound healing in response to H. pylori antigens

The percentages of gastric epithelial cells and fibroblasts migrating to the wound zone are presented on Figure 1A(i) and B(i), respectively, and visualised on images showing the influence of selected antigens which interfered with cell migration and wound healing process [Figure 1A(ii) and B(ii)].

The motility of untreated AGS cells increased with time and the percentages of cells migrating to the "wounded zone" were: 62.3%, 80.8% and 100% after 24, 48 and 72 h, respectively. The rate of wound healing accelerated after 24-h of the cell cultures treatment with GE (10 μg/mL), UreA (5 μg/mL), CagA (1 μg/mL), and *H. pylori* LPS as well as *E. coli* LPS at 1 ng/mL ($P = 0.03$) (Figure 1A). On the other hand, *E. coli* LPS at 25 ng/mL affected cell migration up to 45.4% confluence and 46.1% in 24 and 48-h cell cultures, respectively. By comparison, *H. pylori* LPS at the same concentration (25 ng/mL) inhibited completely the wound healing in 24-, 48- and 72-h cell cultures, which was correlated with a decrease in the cell adhesion (100% lack of confluence). This effect was abolished in the cell cultures exposed for 24 h, but not in those exposed for 48 and 72 h, to *H. pylori* LPS (25 ng/mL) in combination with CagA. Prolonged cell exposure to *H. pylori* LPS (25 ng/mL) and CagA resulted in a complete loss of cell adherence. Interestingly, the inhibitory effect of *H. pylori* LPS on cell migration was abolished in the cell cultures exposed for 24, 48 and 72 h to *H. pylori* LPS (25 ng/mL) in the presence of CagA, UreA and GE.

The results indicate that *H. pylori* compounds differ in terms of their impact on cell migration. *H. pylori* LPS at a high concentration inhibited wound healing, while GE, UreA, CagA as well as *H. pylori* LPS at a low dose accelerated cell motility.

Similarly to AGS cells, the wound healing rate of untreated fibroblasts increased with the time by 10.8%, 42.8% and 68.7% in 24-, 48- and 72-h cell cultures, respectively (Figure 1B). Cell migration increased in cell cultures exposed to UreA ($P = 0.03$) for 24 and 48 h as well as to CagA ($P = 0.03$) or GE ($P = 0.03$) for 24, 48 and 72 h. In contrast, 24, 48 and 72-h incubation of cell cultures with *H. pylori* LPS at 1 ng/mL resulted in a significant decrease in the extent of wound recovery. By comparison, *E. coli* LPS at 1 ng/mL did not affect cell migration 24 and 48 h after the challenge. The percentage of wound confluence was even higher in 72-h cell cultures exposed to the same concentration of *E. coli* LPS as compared to untreated cells. The percentage of wound confluence in 24-h cell cultures, but not in 48- and 72-h cell cultures exposed to *H. pylori* LPS at 25 ng/mL slightly increased when *H. pylori* LPS (25 ng/mL) was combined with CagA. Also, a mixture of *H. pylori* LPS (25 ng/mL) with the following antigens: CagA, UreA and GE minimized the loss of cell confluence. However, these *H. pylori* antigens were not able to completely neutralize the effect of *H. pylori* LPS used at high concentration (25 ng/mL).

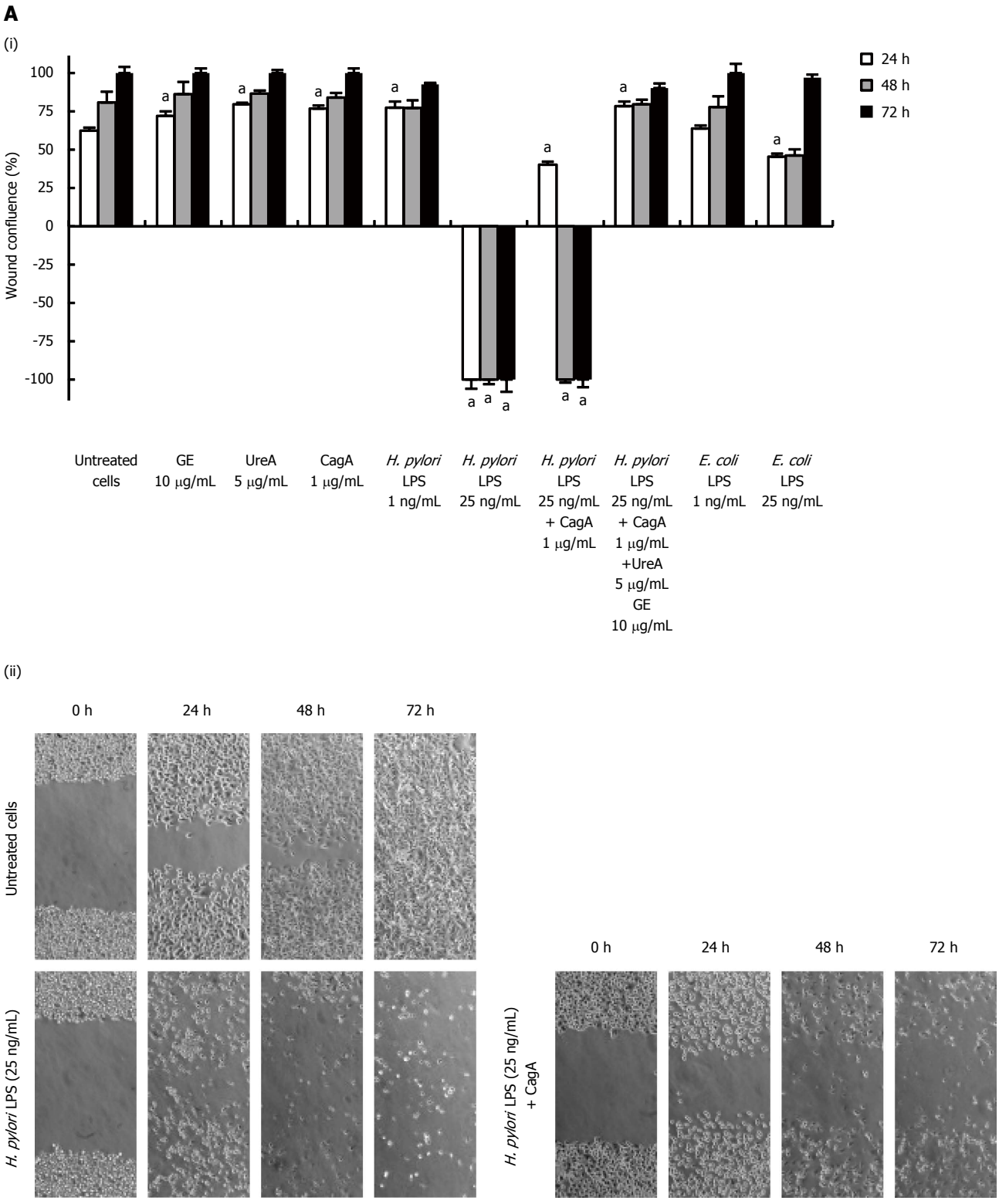
These results show inhibitory effect of *H. pylori* LPS used both at high and low concentrations on the wound healing process in fibroblasts and up-regulation of cell migration after the challenge with other *H. pylori* compounds used separately in the study.

Cell migration vs cell proliferation, cell cycle and metabolic activity in response to H. pylori antigens 24 h after the challenge

In order to specify the mechanism of wound healing in response to activity of *H. pylori* antigens, we compared the impact of *H. pylori* compounds on proliferation, cell viability and cell cycle of AGS cells and fibroblasts 24 h after the challenge. The increased wound closure observed in AGS cell cultures exposed to GE, UreA, CagA or *H. pylori* LPS (1 ng/mL) (Figure 1A) was related to the increased proliferation ($P = 0.03$; Figure 2A) and enhanced ability of the cells to reduce MTT ($P = 0.02$; Figure 2B). The *E. coli* LPS at 1 ng/mL did not influence the cell movement (Figure 1A), proliferation (Figure 2A) or viability (Figure 2B). Despite the reduced wound confluence in response to *E. coli* LPS at 25 ng/mL (Figure 1A), the proliferative activity and cell viability were not affected (Figure 2A and B). By comparison, *H. pylori* LPS at 25 ng/mL downregulated cell migration (100% lack of cell confluence, $P = 0.03$; Figure 1A) and proliferation (SI = 0.3, $P = 0.03$; Figure 2A) as well as cell viability ($P = 0.02$; Figure 2B). A similar effect was observed when *H. pylori* LPS (25 ng/mL) was used in combination with CagA (SI = 0.5, $P = 0.03$). In these conditions also the cell ability to reduce MTT was reduced ($P = 0.02$; Figure 2B). However, when *H. pylori* LPS (25 ng/mL) was used together with CagA, UreA and GE, it lost its inhibitory potential in terms of cell migration as well as proliferative and metabolic activity ($P < 0.05$; Figure 1A, Figure 2A and B).

The obtained results indicate that the ability of *H. pylori* LPS at a high concentration to reduce cell migration is associated with the inhibition of cell spreading and metabolic activity. By comparison, epithelial cells recovered their activity after the exposure to *H. pylori* GE, UreA, CagA or *H. pylori* LPS used at a low dose.

To determine whether the impaired ability of AGS cells to proliferate was related to the cell cycle arrest, we analyzed the cell cycle phase distribution in 24-h cell cultures untreated or pulsed with *H. pylori* antigens. Cells were stained with PI and subjected to


A
(i)
Wound confluence (%)
100
75
50
25
0
-25
-50
-75
-100
24 h
48 h
72 h
a
Untreated cells
GE 10 μg/mL
UreA 5 μg/mL
CagA 1 μg/mL
H. pylori LPS 1 ng/mL
H. pylori LPS 25 ng/mL
H. pylori LPS 25 ng/mL + CagA 1 μg/mL
H. pylori LPS 25 ng/mL + CagA 1 μg/mL +UreA 5 μg/mL GE 10 μg/mL
E. coli LPS 1 ng/mL
E. coli LPS 25 ng/mL
(ii)
0 h
24 h
48 h
72 h
Untreated cells
H. pylori LPS (25 ng/mL)
H. pylori LPS (25 ng/mL) + CagA

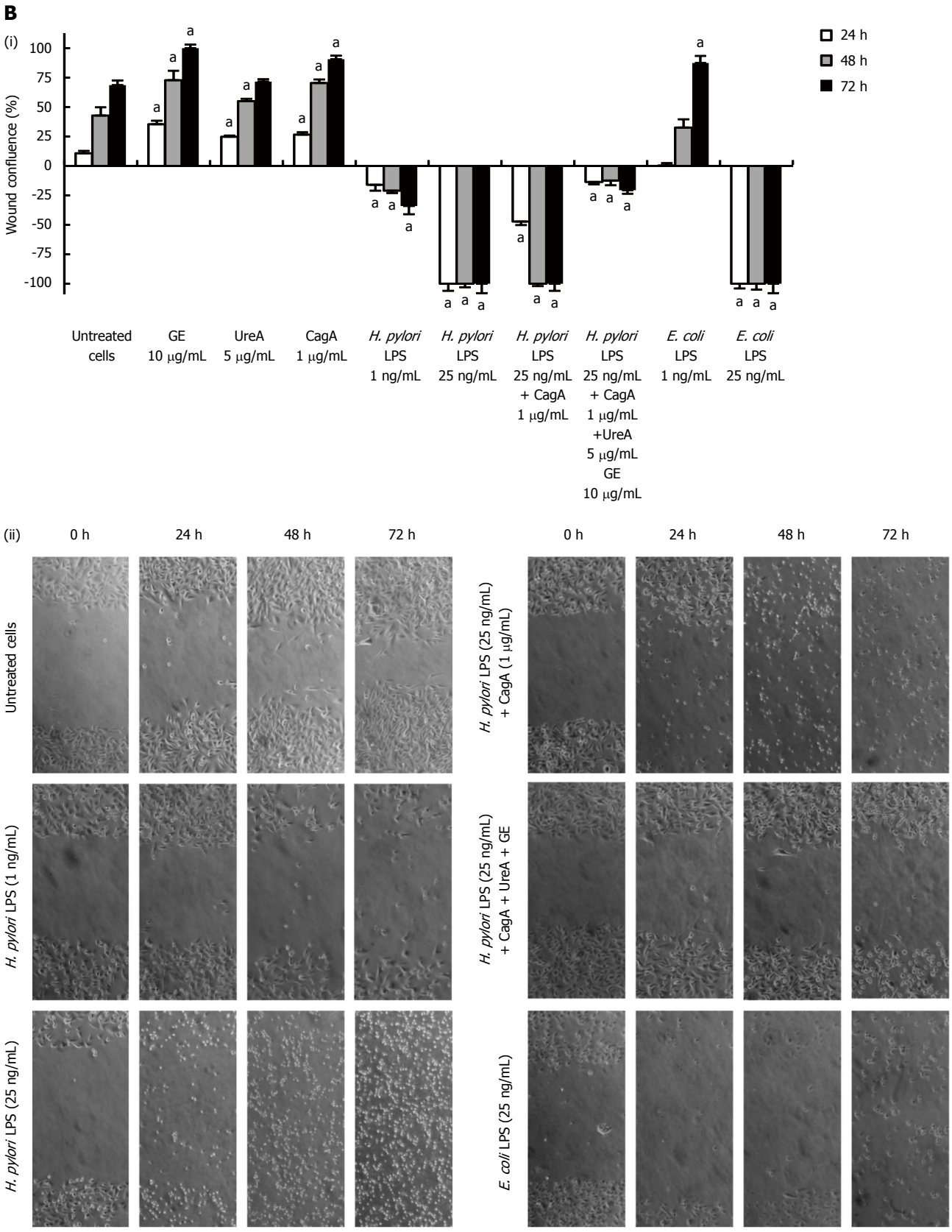


Figure 1 Migration effectiveness of human epithelial AGS cells and guinea pig fibroblasts assessed in a scratch assay. (i) AGS cells (A) and fibroblasts (B) were grown to confluence and incubated overnight in RPMI-1640 medium/2% FBS/1% standard antibiotics. A wound was then made in a cell monolayer and culture medium alone or solutions of bacterial antigens were added. Wound areas were measured at 0, 24, 48 and 72 h after the challenge. Graphs of the average wound size against time, in which the results are shown for cells incubated alone (culture medium) or treated with GE (10 μg/mL), UreA (5 μg/mL), CagA (1 μg/mL) and *Helicobacter pylori* (*H. pylori*) LPS as well as *Escherichia coli* (*E. coli*) LPS (1 ng/mL or 25 ng/mL) or with a combination of *H. pylori* compounds: *H. pylori* LPS (25 ng/mL) and CagA (1 μg/mL) or *H. pylori* LPS (25 ng/mL), CagA (1 μg/mL), UreA (5 μg/mL) and GE (10 μg/mL). $P = 0.03$ *vs* untreated cells; (ii) Phase-contrast microscopy images were taken at the indicated time points and the extent of wound closure for each treatment variant was calculated as a percentage of migrating cells. Representative photos of each time point are shown (magnification × 200). [a]$P = 0.03$ *vs* untreated cells (according to the time of stimulation).

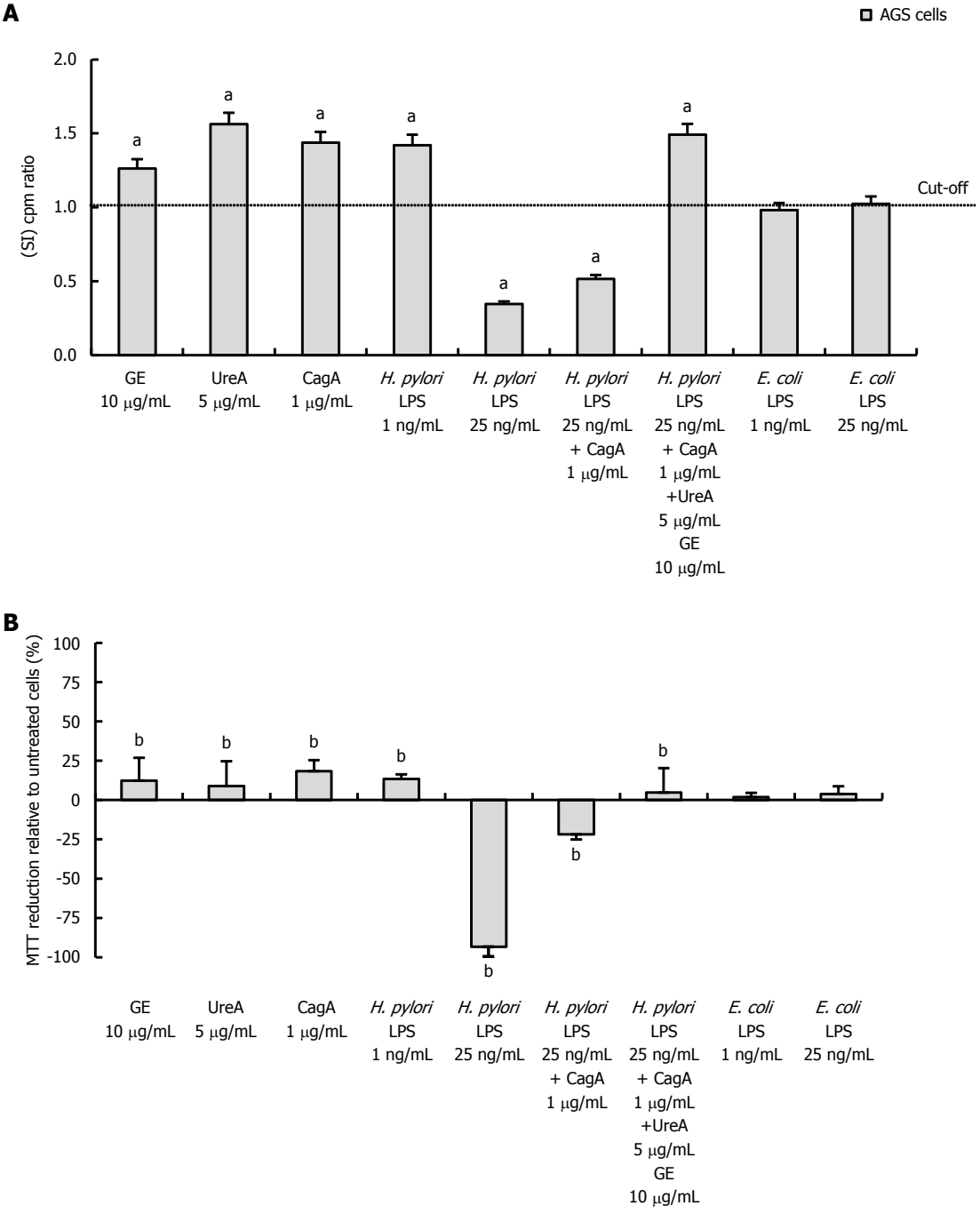


Figure 2 Influence of bacterial antigens on AGS cell proliferation and ability to reduce MTT. A: The proliferative activity of AGS cells was estimated in cell cultures non-stimulated or stimulated for 24 h with bacterial antigens. After incubation, [^{3}H]-thymidine incorporation into cellular DNA was analyzed. The graph shows the stimulation index (SI), which was calculated by dividing the radioactivity counts (cpm/min) for the cell cultures in the presence of a stimulus by the counts for control cell cultures in RPMI-1640 medium alone. The results are shown as SI ± SD of six independent experiments, performed in triplicates. [a]*P* = 0.03 *vs* untreated cells; B: AGS were treated for 24 h with bacterial antigens. After incubation, the ability of cells to reduce MTT was estimated. The graph shows the percentage of MTT reduction ± SD relative to untreated cells. The data represent the average values of four independent experiments performed in triplicates. The values have been normalized to those of the untreated cells. [b]*P* = 0.02 *vs* untreated cells.

flow cytometric analysis. As shown in Figure 3, 63%, 21% and 13% of untreated AGS cells were in the G_1, S and G_2 phase, respectively. It was shown that *H. pylori* LPS at 25 ng/mL, 24 h after the challenge, prevented AGS cells from entering the G_2 phase, resulting in the accumulation of AGS cells in the S phase (37%, *P* = 0.01). Similarly, *H. pylori* LPS (25 ng/mL) in the presence of CagA blocked the cell cycle and caused an increase in the cell number in the S phase (34%), (*P* = 0.03). A mixture of *H. pylori* LPS (25 ng/mL), CagA, UreA and GE did not induce any block in the cell turnover. By comparison, *H. pylori* LPS at 1 ng/mL and *E. coli* LPS at both low and high concentrations as well as other *H. pylori* antigens used separately did not affect the cell cycle.

These results reveal that the disturbance in epi-

	Cell cycle of AGS cells			
	G_1	S	G_2	Apoptosis
Untreated cells	63%	21%	13%	3%
GE (10 μg/mL)	64%	20%	14%	2%
UreA (5 μg/mL)	62%	22%	15%	1%
CagA (1 μg/mL)	61%	20%	16%	3%
H. pylori LPS (1 ng/mL)	62%	22%	15%	1%
H. pylori LPS (25 ng/mL)	49%	**37**% P = 0.01	9%	5%
H. pylori LPS (25 ng/mL) + CagA (1 μg/mL)	50%	**34**% P = 0.03	7%	9%
H. pylori LPS (25 ng/mL) + CagA (1 μg/mL) + UreA (5 μg/mL) + GE (10 μg/mL)	67%	14%	12%	7%
E. coli LPS (1 ng/mL)	53%	22%	12%	13%
E. coli LPS (25 ng/mL)	63%	20%	13%	2%

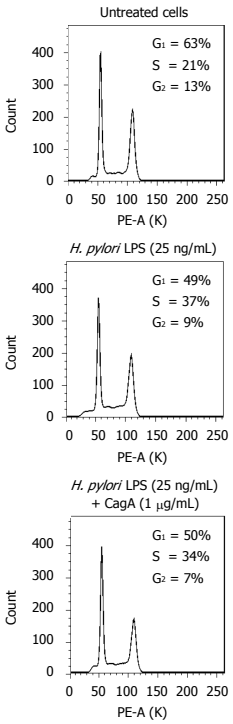


Figure 3 Effect of *Helicobacter pylori* antigens on the epithelial cell cycle profile. AGS cells were incubated for 24 h in RPMI-1640 medium alone or in the presence of bacterial antigens. The cell cycle profile was determined by propidium iodide (PI) staining and the analysis was performed by flow cytometry. The data represent the percentage of cells in each cycle phase of six experiments. Statistically significant differences are indicated as $P < 0.05$ *vs* untreated cells and included in DNA histograms.

thelial cell migration induced by high dose of *H. pylori* LPS is a result of the cell cycle arrest.

The similar comparisons were made for the wound healing capacity of fibroblasts (Figure 1B), their proliferative activity (Figure 4A), the ability to reduce MTT (Figure 4B) and cell cycle distribution (Figure 5), in response to *H. pylori* antigens 24 h after the challenge. As shown in Figure 1B, migration of fibroblasts was modulated positively or negatively depending on the *H. pylori* antigen. The percentage of wound closure increased in response to single *H. pylori* antigens: GE, UreA or CagA, 24 h after the challenge (Figure 1B). In the case of GE, this effect was related to proliferation enhancement (P = 0.01) and MTT reduction (P = 0.02), whereas in response to UreA both activities were on the level of untreated cells (Figure 4A and B). In the cell cultures exposed to CagA the enhanced MTT reduction was shown (P = 0.01, Figure 4B). Reduced cell migration (-16%), which was observed in response to *H. pylori* LPS at 1 ng/mL, was not related to a decrease in MTT reduction and cell proliferation (Figure 1B, Figure 4A and B). However, even at the low concentration, *H. pylori* LPS caused an accumulation of cells in the S phase (25%), (P = 0.05; Figure 5). The strongest effect on fibroblasts was induced by *H. pylori* LPS at 25 ng/mL. At this concentration, *H. pylori* LPS completely abrogated the process of wound healing (Figure 1B), significantly diminished the proliferative activity of the cells, (P = 0.02; Figure 4A), and MTT reduction (P = 0.001; Figure 4B). This phenomenon was related to the increased number (16%) of cells in the G_2 phase of the cell cycle (P = 0.02; Figure 5). It was also shown that *H. pylori* LPS at 25 ng/mL even in combination with CagA downregulated cell migration (P = 0.03; Figure 1B), which was followed by the inhibition of proliferative response (P = 0.02; Figure 4A), MTT reduction, (P = 0.001; Figure 4B) and the cell cycle arrest in the S phase (28%), (P = 0.03; Figure 5). Stimulation of fibroblasts with the combination of *H. pylori* compounds (CagA, UreA, GE) in the presence of *H. pylori* LPS at 25 ng/mL resulted in the inhibition of the cell functions: migration (P = 0.03; Figure 1B), proliferation (P = 0.02; Figure 4A), MTT reduction (P = 0.001; Figure 4B) and the cell cycle arrest in the S phase (21%, P = 0.05; Figure 5). However, none of these antigens used alone (without *H. pylori* LPS at 25 ng/mL) affected any of these functions. By comparison, standard *E. coli* LPS at 1 ng/mL did not affect cell migration, proliferation or metabolic activity, whereas at 25 ng/mL it downregulated the cell movement (P = 0.03; Figure 1B), proliferation (P = 0.02; Figure 4A) and MTT reduction (P = 0.00002; Figure 4B). These effects were associated with the cell

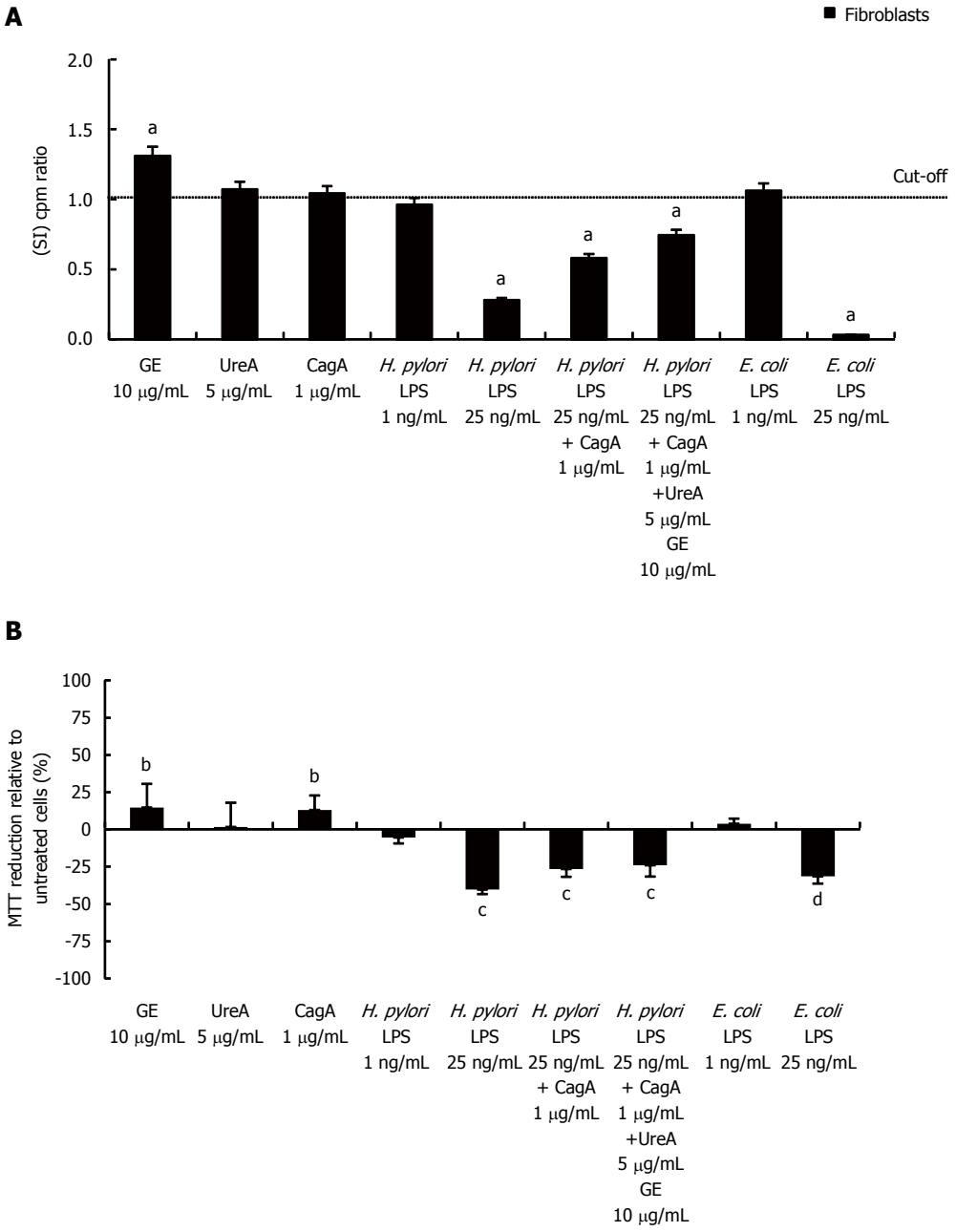


Figure 4 Influence of bacterial antigens on the ability of fibroblasts to proliferate and reduce MTT. A: The proliferative activity of fibroblasts was estimated in cell cultures non-stimulated or stimulated for 24 h with bacterial antigens. After incubation, [^{3}H]-thymidine incorporation into cellular DNA was analyzed. The graph shows the stimulation index (SI), which was calculated by dividing the radioactivity counts (cpm/min) for the cell cultures in the presence of a stimulus by the counts for control cell cultures in RPMI-1640 medium alone. The results are shown as SI ± SD of six independent experiments, performed in triplicates. $^aP = 0.02$ *vs* untreated cells; B: Fibroblasts were treated for 24 h with bacterial antigens. After incubation, the ability of cells to reduce MTT was estimated. The graph shows the percentage of MTT reduction ± SD relative to untreated cells. The data represent the average values of four independent experiments performed in triplicates. The values have been normalized to those of the untreated cells. $^bP = 0.01$; $^cP = 0.001$; $^dP = 0.00002$ *vs* untreated cells.

cycle arrest (17%) in the G_2 phase, $P = 0.01$ (Figure 5).

These studies show that fibroblasts similarly to the epithelial cells are sensitive to high concentration of *H. pylori* LPS, but in contrast to epithelial cells fibroblasts are also affected by low concentration of *H. pylori* LPS as well as high dose of *E. coli* LPS.

H. pylori LPS-induced cell dysfunction vs DNA disintegration, cell apoptosis or necrosis

The results presented above prompted us to search for a deeper explanation of the nature of *H. pylori* LPS-dependent negative modulation of the cell functions, on the level of DNA integrity and signs of cell death. To examine whether *H. pylori* LPS at 25 ng/mL may induce DNA damage in AGS cells and fibroblasts, we performed DAPI staining and a comet assay. The results of these assays are shown in Figure 6A and B for AGS cells and fibroblasts, respectively. *H. pylori* LPS at 25 ng/mL and in combination with CagA (but not CagA alone) induced significant DNA condensation

	Cell cycle of fibroblasts			
	G1	S	G2	Apoptosis
Untreated cells	72%	16%	11%	1%
GE (10 μg/mL)	71%	15%	13%	1%
UreA (5 μg/mL)	69%	18%	11%	2%
CagA (1 μg/mL)	71%	12%	15%	2%
H. pylori LPS (1 ng/mL)	63%	25% *P* = 0.05	12%	0%
H. pylori LPS (25 ng/mL)	68%	12%	16% *P* = 0.02	4%
H. pylori LPS (25 ng/mL) + CagA (1 μg/mL)	58%	28% *P* = 0.03	11%	3%
H. pylori LPS (25 ng/mL) + CagA (1 μg/mL) + UreA (5 μg/mL) + GE (10 μg/mL)	67%	21% *P* = 0.05	11%	1%
E. coli LPS (1 ng/mL)	65%	20%	11%	4%
E. coli LPS (25 ng/mL)	69%	13%	17% *P* = 0.01	1%

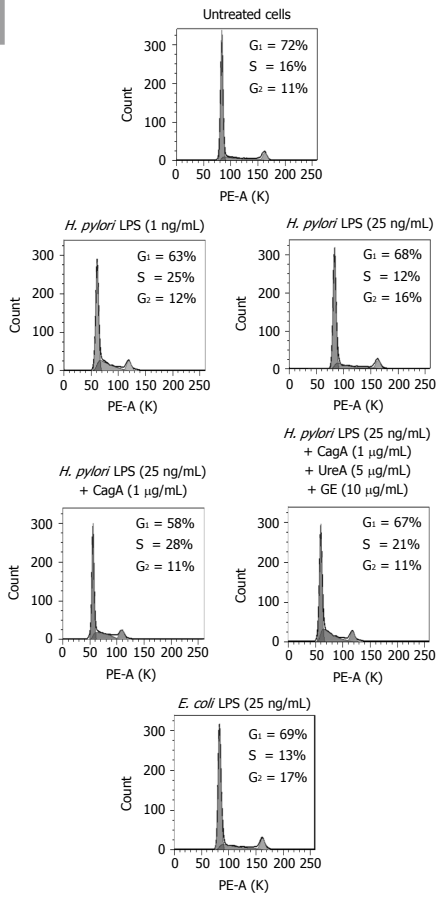


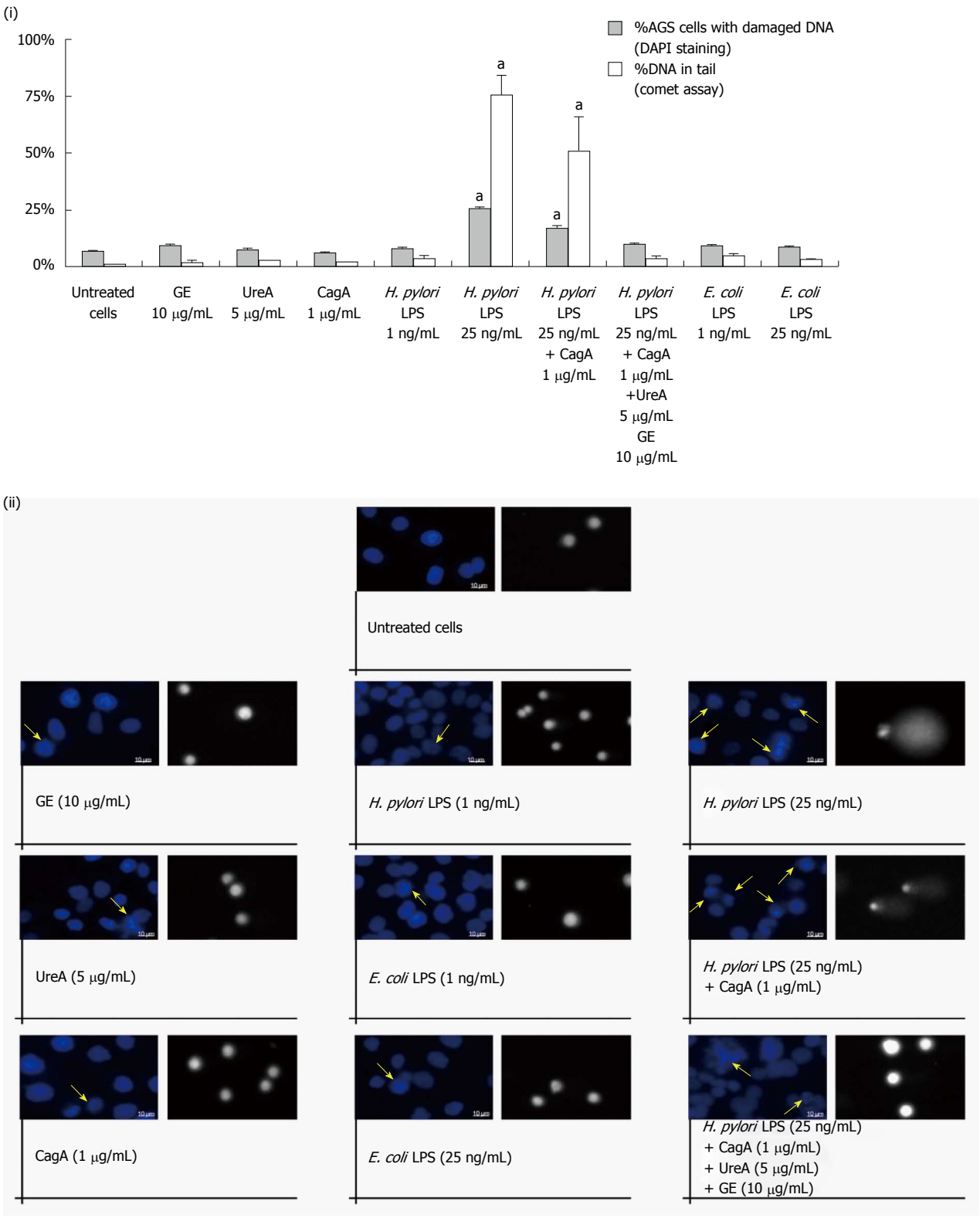
Figure 5 Effect of *Helicobacter pylori* antigens on the fibroblast cell cycle profile. Fibroblasts were incubated for 24 h in RPMI-1640 medium alone or in the presence of bacterial antigens. The cell cycle profile was determined by propidium iodide (PI) staining and the analysis was performed by flow cytometry. The data represent the percentage of cells in each cycle phase of six experiments. Statistically significant differences are indicated as $P < 0.05$ *vs* untreated cells and included in DNA histograms.

in 24.7% and 16.3% of AGS cells, respectively, which was shown by the percentage of cells with damaged DNA and visualized by an increased fluorescence intensity (Figure 6A). Furthermore, *H. pylori* treatment led to longer DNA smears: 75% and 49.7% DNA in tail, respectively ($P < 0.0001$; Figure 6A).

In a cell culture of fibroblasts, *H. pylori* LPS induced dose dependent DNA damage as assessed by DAPI staining and a comet assay (Figure 6B). The percentages of fibroblasts treated with *H. pylori* LPS at 25 ng/mL or 1 ng/mL with the signs of DNA damage detected by DAPI staining were 45.7% and 12.7%, respectively ($P = 0.03$). A higher concentration of *H. pylori* LPS (25 ng/mL) caused longer DNA smears, whereas a lower concentration (1 ng/mL) resulted in the formation of shorter comet tails, 78.7% ($P = 0.000001$) and 6.4% ($P = 0.0001$), respectively (Figure 6B). DNA damage was also detected in cell cultures treated with *H. pylori* LPS (25 ng/mL) and CagA (23.7%, $P = 0.03$) or *H. pylori* LPS (25 ng/mL) and other *H. pylori* compounds: CagA, UreA and GE (14%, $P = 0.03$). However, cellular DNA damage induced by a combination of *H. pylori* antigens was lower than in the cells treated with *H. pylori* LPS (25 ng/mL) alone (45.7%, $P = 0.03$). All these results are in accordance with the results obtained in a comet assay (Figure 6B). By comparison, in cell cultures treated with standard *E. coli* LPS at 25 ng/mL DNA damage was detected in 24% fibroblasts ($P = 0.03$) and the percentage of DNA in the tail was 72.4%, $P < 0.05$ (Figure 6B). *E. coli* LPS at 1 ng/mL did not induce any significant DNA damage.

In order to clarify whether the reduction of cells viability, and the inhibition of cell migration are related to apoptosis or necrosis, FACS analysis was performed. The results are presented in Figure 7A and B for AGS cells and fibroblasts, respectively. AGS cells were cultured with or without *H. pylori* LPS (25 ng/mL) or *H. pylori* LPS in combination with CagA for 24 h and subjected to flow cytometry analysis after staining with Ann-V-FITC and PI. As shown in Figure 7A, 91% of uninfected AGS cells were viable (Ann-V^- PI^-), 3% were early apoptotic, 4% were late apoptotic (Ann-V^+ PI^-) and 2% were necrotic (Ann-V^- PI^+). In cell cultures of AGS treated with *H. pylori* LPS alone at 25 ng/mL an increase in the percentage of cells undergoing

A


(i)
100%
75%
50%
25%
0%
%AGS cells with damaged DNA (DAPI staining)
%DNA in tail (comet assay)
a
a
a
a
Untreated cells
GE 10 μg/mL
UreA 5 μg/mL
CagA 1 μg/mL
H. pylori LPS 1 ng/mL
H. pylori LPS 25 ng/mL
H. pylori LPS 25 ng/mL + CagA 1 μg/mL
H. pylori LPS 25 ng/mL + CagA 1 μg/mL +UreA 5 μg/mL GE 10 μg/mL
E. coli LPS 1 ng/mL
E. coli LPS 25 ng/mL
(ii)
Untreated cells
GE (10 μg/mL)
H. pylori LPS (1 ng/mL)
H. pylori LPS (25 ng/mL)
UreA (5 μg/mL)
E. coli LPS (1 ng/mL)
H. pylori LPS (25 ng/mL) + CagA (1 μg/mL)
CagA (1 μg/mL)
E. coli LPS (25 ng/mL)
H. pylori LPS (25 ng/mL) + CagA (1 μg/mL) + UreA (5 μg/mL) + GE (10 μg/mL)

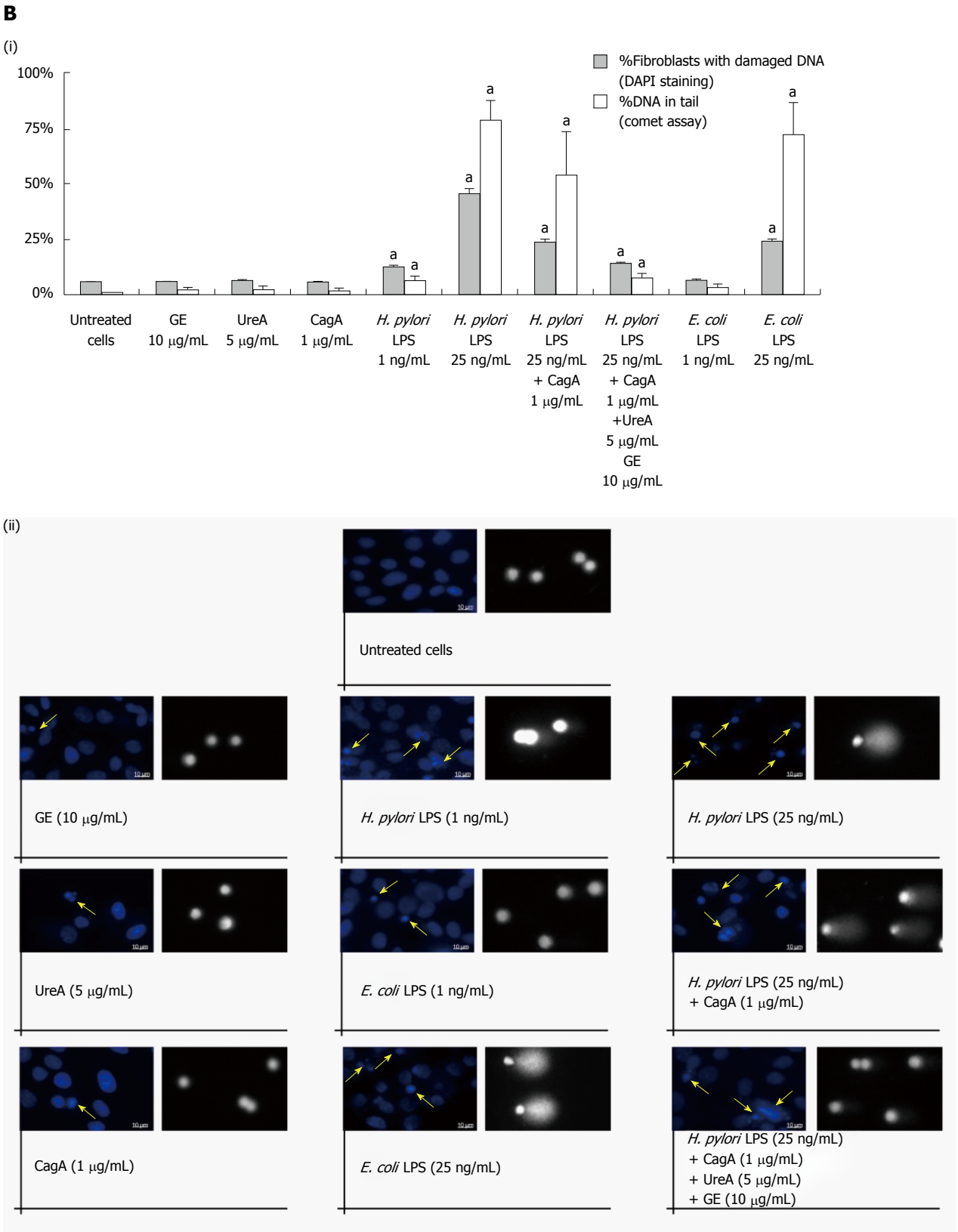


Figure 6 Genotoxic properties of *Helicobacter pylori* antigens assessed by 4',6-diamidino-2-phenylindole staining and a comet assay. The influence of *Helicobacter pylori* or *E. coli* antigens on DNA stability was estimated in AGS cells (A) and fibroblasts (B) 24 h after the challenge. A 4',6-diamidino-2-phenylindole (DAPI) staining assay was used to visualize DNA changes in cell nuclei and a comet assay was applied to confirm DNA damage by the measurement of the percentage of DNA in the comet tail. Mean values were replicated of 50 comets each. The values are the means ± SD. $^{a}P < 0.05$ *vs* untreated cells. (i) the graphs indicate the percentage of cells with DAPI stained nuclei (blue bars) and the percentage of nuclei with DNA in the comet tail (grey bars); and (ii) visualization of morphological changes in the cell nuclei after the treatment with bacterial antigens followed by DAPI staining and a comet assay. Imaging was performed using a fluorescent microscope (Axio Scope A1, Zeiss, Germany). The arrows indicate the damaged cell nuclei (magnification, × 1000). DNA tails were measured using the CASP software (latest beta version 1.2.3.beta2). Representative results of the comet assay were selected (magnification, × 400).

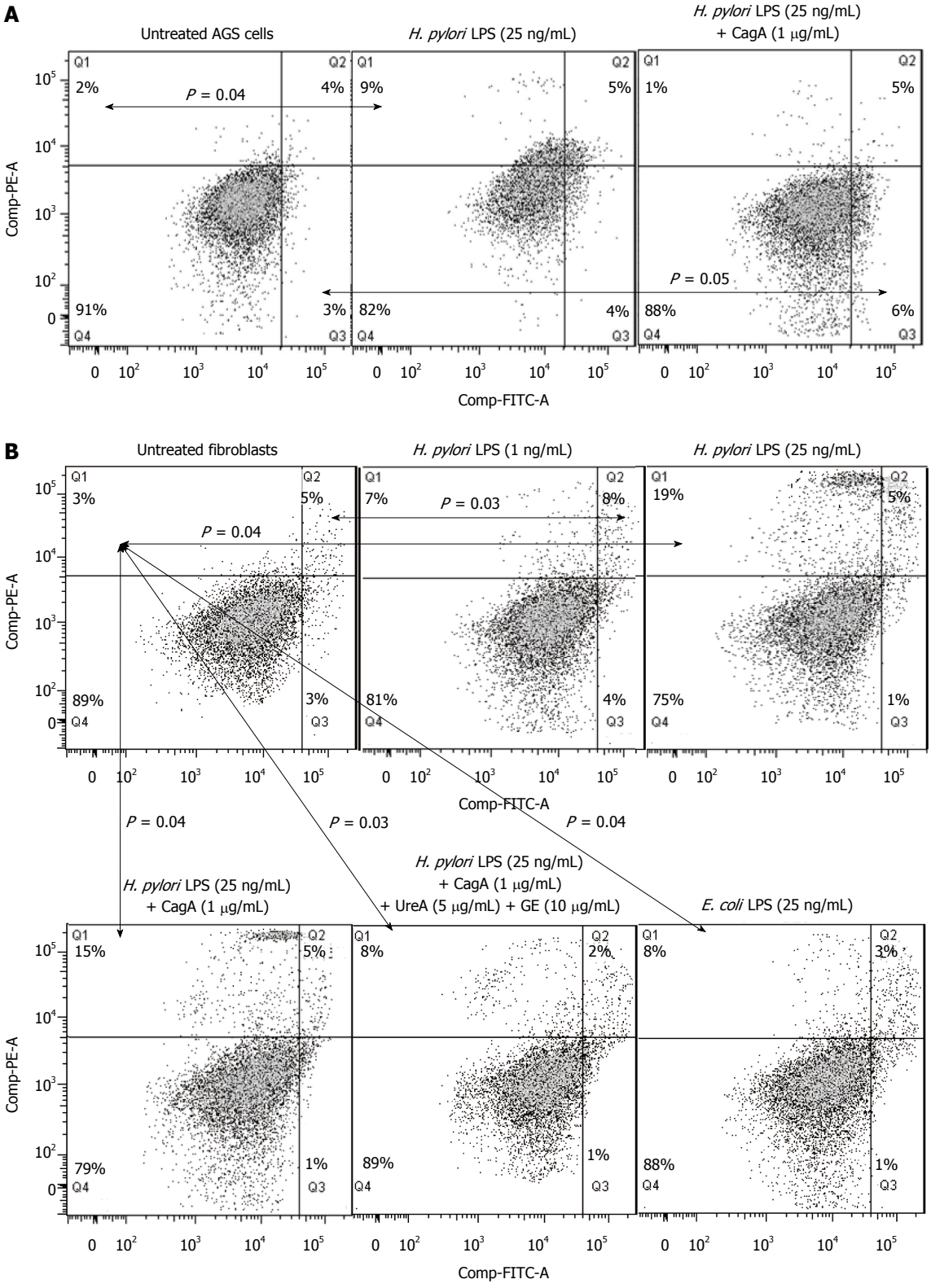


Figure 7 Type of cell death in response to *Helicobacter pylori* antigens. Effects of bacterial antigens on AGS cells (A) and fibroblasts (B) concerning cell death were measured 24 h after the challenge by double staining of the cells with isothiocyanate fluorescein (FITC)-conjugated annexin V and propidium iodide (PI) using flow cytometry. Quadrants were designed as follows, Q4: Ann-V$^-$/PI$^-$ - viable cells; Q3: Ann-V$^+$/PI$^-$ - cells with the signs of early apoptosis; Q2: Ann-V$^+$/PI$^+$ - cells with the signs of late apoptosis; Q1: Ann-V$^-$/PI$^+$ - necrotic cells. All dot plots are a representation of equal cell populations (the fluorescence of 10000 cells was gated and counted using the FlowJo software). The data represent the average values of six independent experiments. Statistically significant differences are indicated as $P < 0.05$ *vs* untreated cells.

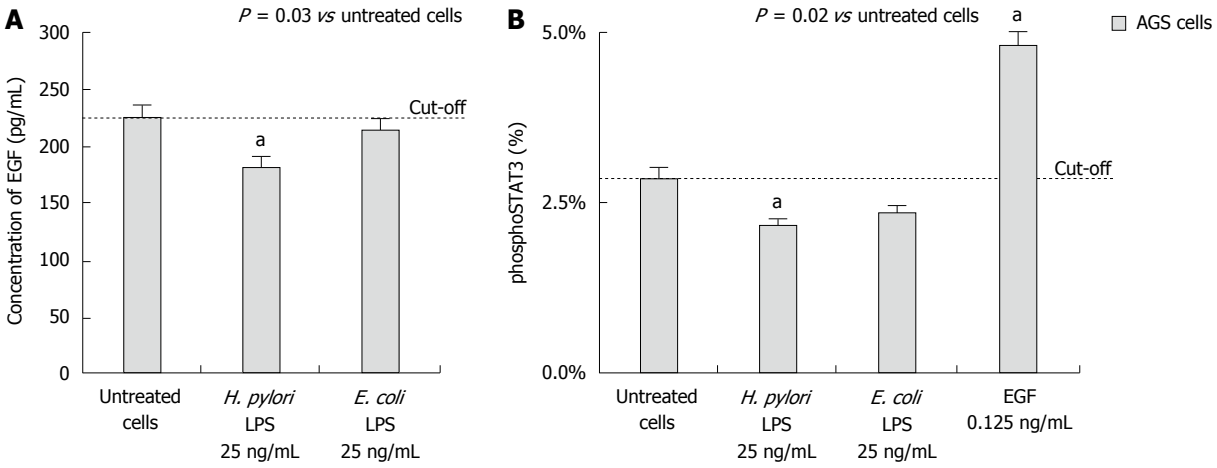


Figure 8 *Helicobacter pylori* lipopolysaccharide-driven inhibition of signal transducer and activator of transcription 3 phosphorylation and epidermal growth factor production. A: The impact of *Helicobacter pylori* or *E. coli* lipopolysaccharide (LPS) on the secretion of epidermal growth factor (EGF) by AGS cells; B: the percentage of phospho-signal transducer and activator of transcription 3 (phosphoSTAT3) in AGS cells measured by cell-based ELISA. Cells treated with EGF (0.125 ng/mL) were used as a positive control. The *cut-off* value was related to the response of untreated cells. The data represent the average values of four independent experiments. Statistically significant differences are indicated as $^{a}P < 0.05$ *vs* untreated cells.

necrosis was observed for 9% of all cells as compared to untreated cells, $P = 0.04$. However, there were no significant differences between the percentages of early and late apoptotic cells. When the cells were treated with the combination of *H. pylori* LPS (25 ng/mL) and CagA, an increased number of early apoptotic cells was detected (6%). In comparison to untreated cells with signs of early apoptosis (3%) this difference was of low significance $P = 0.05$.

As shown in Figure 7B, 89% untreated fibroblasts were viable (Ann-V$^-$ PI$^-$), 5% were late apoptotic and 3% were necrotic. Nevertheless, 8% fibroblasts showed the signs of late apoptosis ($P = 0.03$) after a treatment with *H. pylori* LPS at 1 ng/mL. In the cell cultures incubated in the milieu of *H. pylori* LPS at 25 ng/mL, only 75% fibroblasts were viable, while 19% underwent necrosis ($P = 0.04$). In the presence of standard *E. coli* LPS at 25 ng/mL, 8% of fibroblasts underwent necrosis ($P = 0.04$). Challenging the cells with *H. pylori* LPS (25 ng/mL) in combination with CagA or CagA, UreA and GE resulted in a decreased percentage of necrotic fibroblasts (15%, $P = 0.04$ and 8%, $P = 0.03$, respectively).

H. pylori LPS induces a decrease in EGF secretion and phosphorylated Y(705)STAT3 concentration in gastric epithelial cells

The Janus kinase (JAK)/STAT3 pathway is one of the major signal transduction pathways. STAT3 is activated through phosphorylation in response to various cytokine and growth factors including EGF. STAT3 mediates the expression of a variety of genes in response to cell stimuli, and thus plays a key role in many cellular processes such as cell growth and apoptosis. In order to specify the mechanism of *H. pylori* LPS induced inhibition of wound healing, we examined the ability of the cells to secrete EGF in relation to the concentration of phosphorylated STAT3. For this purpose we used supernatants obtained from AGS cell cultures untreated or stimulated with bacterial antigens. Only, in response to *H. pylori* LPS at 25 ng/mL, the concentration of EGF was decreased as compared to untreated cells ($P = 0.03$; Figure 8A). The decrease in EGF secretion was correlated with a reduced amount of phospho-STAT3 ($P = 0.02$; Figure 8B). Other bacterial antigens did not cause a decrease in the concentration of EGF and phospho-STAT3 (data not shown).

DISCUSSION

H. pylori colonization of the gastric tissue promotes excessive inflammation, which exerts harmful effects on the gastric mucosa. However, precise mechanisms of tissue destruction are not well understood. *H. pylori* produce many virulence factors responsible for cell damage, which facilitate the survival of these pathogens on the surface of gastric mucosa and evasion of the immune response of the host[74-77]. In this study, we used human gastric epithelial AGS cells and guinea pig fibroblasts as an *in vitro* model for the assessment of the effects of interactions between *H. pylori* and the gastric epithelial barrier, which might also take place *in vivo*. Gastric epithelial cells constitute the first protective barrier responsible for the maintenance of local homeostasis. Fibroblasts were chosen for several reasons: *in vivo* they are present in the sub-epithelial mucosa and can be targeted by *H. pylori* compounds leaking through epithelial lesions or transferred there through the epithelium[78]. Fibroblasts are involved in the wound healing and participate in many immunological processes including direct response to pro-inflammatory cytokines[79,80]. In order to restore gastric epithelial homeostasis, gastric tissue ulceration initiated

via H. pylori - host cell interaction should be followed by a healing process. In this study, we monitored the influence of *H. pylori* antigens on the healing process by the assessment of the wound repair using a scratch assay mimicking an ulcer lesion. The ability of the cells to heal an injury was monitored in relation to cell viability, proliferating activity and the cell cycle as well as genotoxicity of bacterial compounds used for the cell challenge: *H. pylori* GE, which is a complex of surface antigens, UreA urease subunit, CagA protein and LPS. All of these components are present in the environment inhabited by *H. pylori*. In this study, we have shown that *H. pylori* antigens may differ in their effects towards epithelial cells as well as fibroblasts and that both cell types differ in their susceptibility to various *H. pylori* antigens. Epithelial AGS cells after the challenge with GE, UreA and CagA were able to repair the wound, which was associated with an increased number of viable cells and intensified cell proliferation while in fibroblast cultures these parameters were elevated after the challenge with GE and CagA but not with UreA. Increased cell migration and proliferation are necessary to heal tissue damage. It has been suggested that a possible molecular mechanism of wound healing during *H. pylori* infection could be related to *H. pylori*-derived RpL1 aa 2-20 peptide (Hp 2-20), which by interacting with formyl peptide receptors induces cell migration and proliferation, as well as the expression of the vascular endothelial growth factor, thereby promoting gastric mucosal healing[81]. However, Pousa *et al*[82] have suggested that early angiogenesis required for the repair process during *H. pylori* infections is inhibited probably due to the anti-proliferating properties of the bacteria.

While increased proliferation is essential for gastric tissue healing, an uncontrolled proliferative activity can promote the accumulation of harmful mutations and cancerogenesis[83]. The mechanism preventing these processes is apoptosis. In our study, increased proliferation of AGS cells in response to CagA, UreA and GE as well as enhanced proliferation of fibroblasts after the challenge with GE were not accompanied by a parallel increase in cell apoptosis. Several studies have shown that *cagA*$^+$ strains have a greater carcinogenic potential than *cagA*$^-$ strains[30]. Increased cellular proliferation in response to *H. pylori* infection, especially with *cagA*+ strains, has been confirmed *in vivo* both in humans and laboratory animals[84,85]. It has been shown that the functional *cag* secretion system is required for the induction of phosphatidylinositol 3-kinase, an integral component of a signal transduction pathway, which leads to an increase in the cell proliferation and inhibition of apoptosis[86,87]. However, in this study we observed increased epithelial cell proliferation also in response to soluble CagA, which *in vivo* might be present in the inflammatory milieu and translocated to cytosol by phagocytosis or endocytosis. Also, interaction *via* surface receptors cannot be excluded. It is worth mentioning that, while CagA enhances proliferation of epithelial cells, it inhibits the division of peripheral blood *H. pylori*-reactive T lymphocytes possibly allowing these bacteria to survive in the host[31]. *In vivo*, enhanced gastric mucosal proliferation and the low apoptosis rate were positively correlated with the severity of acute gastritis, which means that hyperproliferation not balanced by cell death might contribute to neoplasia[30]. The relation between increased expression of the cellular proliferation marker Ki-67 (also known as MKI67) in gastric tissue sections and severity of inflammatory response has been shown in *H. pylori*-infected guinea pigs[85].

Recently, it has been indicated that not only CagA but also other *H. pylori* compounds such as adhesin BabA and JHP0290 protein, may contribute to *H. pylori*-associated diseases by promoting gastric epithelial cell proliferation and increased resistance to apoptosis[88,89]. In our study elevated gastric epithelial cell expansion was observed after exposure to *H. pylori* GE, UreA and a low dose of *H. pylori* LPS. Moreover, *H. pylori* GE increased also proliferation of fibroblasts. It may lead to either tissue regeneration or neoplasia. In our study, *H. pylori* LPS used at a low concentration (1 ng/mL) accelerated wound repair, which was associated with significantly enhanced cell proliferation and MTT reduction, whereas *H. pylori* LPS at a high concentration (25 ng/mL), inhibited the wound repair process. Also another study revealed that *H. pylori* LPS (1 μg/mL) accelerated the proliferation rate of gastric epithelial cells *via* TLR2, and a MEK-1/2-ERK-1/2 (mitogen-activated protein (MAP) kinase activating extracellular-signal-regulated kinases) MAP kinase cascade[90]. Similar dose-dependent effects were found for *Pseudomonas aeruginosa* LPS in a model of pulmonary epithelial cell damage[91]. AGS cell response to the low concentration of *H. pylori* LPS observed in our study suggests that gastric epithelium is able to react to deleterious signals by the activation of host defence mechanisms. However, this barrier was destabilised after exposure to a high dose of *H. pylori* LPS. In this case, during natural infection, the *in vivo* effects of *H. pylori* on the gastric barrier may depend on the type and local concentration of bacterial compounds. It is worth mentioning that the colonization of *H. pylori* is not homogenous, but is stratified by a gastric site and acuteness of inflammation. This allows increasing the concentrations of the individual on-site components. Interestingly, *E. coli* LPS used at a concentration of 25 ng/mL did not affect the process of wound repair in the AGS cell monolayer. This could be due to the lack of MD2 protein in these cells, which is involved in the recognition of *E. coli* LPS[92]. Therefore, the migration of AGS cells may not be inhibited in response to this type of LPS. Different effect of *H. pylori* LPS could be due to its engagement in the signaling pathway mainly with the participation of TLR2 but not TLR4

host cell receptors[90]. Various activities of *H. pylori* LPS and *E. coli* LPS may be the result of their different chemical structures. *H. pylori* LPS contains Le^x and Le^y determinants, which make the bacteria less visible to the immune cells and therefore it might exert different effects on the level of gastric epithelial cells[43,50,93].

In the case of fibroblasts, which in the natural environment constitute the deeper layers of the gastric mucosa, *H. pylori* LPS impaired wound healing regardless of its concentration. Even an addition to the cell culture of other *H. pylori* antigens (UreA, GE, CagA), which alone intensified the migration of fibroblasts and wound healing, did not result in neutralizing the inhibitory effect of *H. pylori* LPS at a high concentration. The motility and viability of fibroblasts were also inhibited after the challenge of the cells with *E. coli* LPS used at the concentration of 25 ng/mL, indicating that generally fibroblasts are more susceptible to various bacterial LPS than AGS epithelial cells and that similar cell signaling pathways can be involved in the response to different types of LPS. *In vivo*, such deep disruption of the epithelial barrier by *H. pylori* components and inflammatory mediators may facilitate damage to lamina propria, and promote the development of both local and systemic inflammatory response. *H. pylori* antigens penetrating across the gastric epithelium, can be processed in lamina propria by macrophages *via* PRR and presented to T lymphocytes[8,19,20]. However, it has been shown that *H. pylori* LPS is able to downregulate the lymphocyte blastogenic response, probably due to the interference with the process of macrophage maturation[46-48,94]. It also downregulates the phagocytic potential of macrophages[44] and decreases the cytotoxic activity of NK cells[45,68]. In the gastric epithelium, colonized by pathogenic microorganisms, including *H. pylori*, probably IL-33 acting as an alarming molecule can induce the signalling beneficial for tissue recovery due to a short-term increase in endothelial permeability. However, under some circumstances it may cause the aggravation of inflammation and tissue dysfunction by attracting Th2 lymphocytes, promotion of cell apoptosis and maintenance of tissue dysfunction[95]. *In vivo*, during *H. pylori* infection different environmental conditions (*e.g.,* stress, nutrients, pH), time of the cell exposure to bacterial antigens and their concentration may also determine the fate of different cell types. Other inflammatory mediators such as matrix metalloproteinases by suppression of apoptosis and preservation of the cells with damaged DNA may influence the rate of epithelial cell growth or cell loss[96-98].

We have shown that CagA, UreA, GE and *H. pylori* LPS at low concentration increased the proliferative activity of AGS cells, which was not accompanied by a parallel increase in cell apoptosis. Cell viability was not altered by these *H. pylori* antigens. *In vivo* loss of the cell integrity could be attributed to other mechanisms such as a breakdown of tight junctions in *H. pylori* infection[3,99,100]. Furthermore, high-temperature requirement A (HtrA) protease secreted by *H. pylori* as well as inflammatory factors induced by *H. pylori* such as interferon gamma and tumour necrosis factor alpha (TNF-α) might favour bacterial attachment to epithelial cells[101-104]. *In vivo*, recruitment of mesenchymal stem cells (MSC) in response to TNF-α secreted by *H. pylori* colonized epithelial cells is necessary to balance *H. pylori*-related apoptosis. However, MSC fusion with epithelial cells may render them more susceptible to neoplastic transformation[105].

We have demonstrated that *H. pylori* LPS at high concentration reduced viability of both AGS cells and fibroblasts, which was associated with the accumulation of AGS cells in the S and fibroblasts in the G_2 cell cycle phase, and with an increased frequency of cell death. These data provide strong evidence for the cell cycle arrest in response to *H. pylori* LPS and, for the inhibition of the cell growth. However, the suppressed cell proliferation could also be attributed to the cell death apart from to the cell cycle arrest. These effects were not observed when AGS cells were treated with *E. coli* LPS at a high dose, which is consistent with the observations made by Peek *et al*[106]. In our study, *H. pylori* LPS at a high dose inhibited the AGS cell cycle in the S phase even in a combination with CagA, which promoted cell proliferation. In the case of fibroblasts, *H. pylori* LPS or *E. coli* LPS at the high concentration arrested the cell cycle in the G_2 phase, whereas a low dose of *H. pylori* LPS inhibited the cell cycle progression in the S phase. In other studies, the *H. pylori*-induced cell cycle arrest of gastric epithelial cells as well as fibroblasts in the G_1 phase was reported[107-111]. It has been shown that also *H. pylori* L-asparaginase inhibited the cell cycle of gastric epithelial cells and fibroblasts and recurrent infections might influence the cell cycle[109]. It means that cellular effects are antigen-, dose- and cell type-dependent.

In general, cells are arrested in the S phase due to the depletion of the substrates required for DNA synthesis, whereas the entry to mitosis is blocked by the G_2 checkpoint mechanism when DNA is damaged[112]. In our study, we used AGS cells and fibroblasts that were not serum starved (unsynchronized cells) in order to mimic events that occur in the naive gastric mucosa. However, treatment of these cells with *H. pylori* LPS at a high concentration resulted in nuclear morphology changes in both cell types, which were visualised by DAPI staining and a comet assay. It confirmed the genotoxic properties of *H. pylori* LPS used at a higher concentration.

The results obtained in this study and data of other authors indicate that *H. pylori* may initiate damage to gastric epithelium directly through its components such as urease, VacA and LPS[100,113-115]. Several mechanisms can drive epithelial cell damage after the challenge with *H. pylori* LPS. It can be due to direct cytotoxic effect

associated with lipid A binding *via* TLR4/TLR2. Handa *et al*[116] have shown that *H. pylori* LPS increased NADPH oxidase (NOX; nicotinamide adenine dinucleotide phosphate oxidase) and TLR4 expression on gastric epithelial cells leading to elevation of deleterious oxidase stress. In this study we used an Le^{xy} positive *H. pylori* strain. Despite TLR4/TLR2 surface receptor binding, such variants can interact with surface lectins *via* Le carbohydrate moieties[52]. It has been shown that Le positive *H. pylori* variants can bind DC-SIGN C-type lectin, which *in vivo* is present on gastric dendritic cells[41,50].

H. pylori may affect gastric homeostasis also indirectly by the interaction with the angiogenesis process and over-expression of inflammatory response. It has been shown that early angiogenesis, which is necessary for epithelial reconstruction[117], is inhibited during *H. pylori* infection probably due to anti-proliferative and pro-apoptotic activity of the bacteria[82,100,113]. *H. pylori* LPS, when transferred into the sub-epithelial space, can hinder polymorphonuclear leukocyte (PMNL) apoptosis[31,118,119]. In order to prevent PMNL apoptosis and increase the cell survival, epithelial cells secrete pro-inflammatory cytokines such as IL-8 and the granulocyte macrophage colony stimulating factor. However, it is followed by an enhanced epithelial injury due to the excess of proteinases and oxidative stress compounds, which are tolerated by *H. pylori* equipped with neutralizing enzymes[77].

An important aspect of wound repair is the ability of cells to respond to EGF which promotes cell migration and wound healing[120]. It has been shown that treatment of AGS cells with non-phosphorylated CagA protein leads to the activation of the JAK/STAT3 signalling pathway. By comparison, phosphorylated CagA has been observed to alter cell morphology, polarity, growth and activation of β-catenin, which is implicated in cancerogenesis[86,121]. In order to determine whether *H. pylori* LPS-driven inhibition of AGS cell proliferation observed in our study was associated with alternation in EGF concentration we evaluated quantitatively both EGF and phospho-STAT3. A high dose of *H. pylori* LPS has been shown to decrease the amount of EGF and phospho-STAT3 in the epithelial cell cultures. This observation might explain the mechanism of *H. pylori* LPS-mediated disturbance in wound healing process.

CONCLUSION

This study shows that *H. pylori* soluble components may affect the balance between proliferation of surface epithelial and lamina propria cells and cell death. This balance is crucial for the renovation of the epithelium, wound healing and protection against neo-plastic transformation. Our results allow suggesting that *in vivo*, during acute or chronic *H. pylori* infection, various cellular effects might depend on target cells, the bacterial antigen and its concentration. Domination of antigens capable of stimulating cell proliferation such as UreA, CagA or surface antigens (GE) can lead to the epithelial renewal, although their excessive activity may pose an increased risk of developing cancer. In contrast, domination of antigens such as LPS with cytotoxic and anti-proliferative activity towards mucosal cells may promote chronic inflammation, and, in the case of immune cells, inhibition of antibacterial response, resulting in the maintenance of *H. pylori* infection.

COMMENTS

Background

The human gastric mucosal barrier is permanently exposed to various infectious agents and their soluble components. Disruption of this barrier homeostasis results in the inflammatory response and may lead to a variety of pathological effects. Among bacterial pathogens, Gram-negative *Helicobacter pylori* (*H. pylori*) rods play a crucial role in the development of gastritis, gastric and duodenal ulcers and even gastric cancers.

Research frontiers

H. pylori demonstrates affinity to gastric epithelium, resulting with an excessive inflammation, peptic ulcers and cancers. Strong inflammation and metaplasia suggest that *H. pylori* interfere with cell growth and initiate different disorders. The research hotspot is to further clarify the mechanisms used by *H. pylori* to maintenance chronic infection.

Innovations and breakthroughs

The authors found that *H. pylori* soluble antigens such as subunit A of urease (UreA), cytotoxin associated gene A protein (CagA) and glycine acid extract antigenic complex (GE) are capable to stimulate cell proliferation leading to the epithelial renewal, although their excessive activity *in vivo* may increase the risk of cancer development. In contrast, domination of antigens such as *H. pylori* lipopolysaccharide (LPS) with cytotoxic and anti-proliferative activity towards mucosal cells may promote chronic inflammation and the maintenance of *H. pylori* infection.

Applications

The results of this study improve the knowledge about the mechanisms used by *H. pylori* to maintenance chronic infection and disrupt barrier function of gastric mucosa.

Terminology

Two independent cell lines: gastric epithelial AGS cells and fibroblasts (challenged with *H. pylori* soluble antigens), were used by the authors in terms of mimicking the interaction of *H. pylori* compounds with gastric mucosal barrier.

Peer-review

This *in vitro* cellular study provides new data about the impact of *H. pylori* soluble antigens such as CagA, UreA, GE and LPS to the gastric mucosal barrier.

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