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The Effect of *Helicobacter pylori* on Apoptosis of the GES-1 Transfected pEGFP-N1-UreB Eukaryotic Expression Plasmid *in vitro*

[1]J.G. Chen, [1]A.X. Liang, [1,2]L. Han, [1]J.J. Xiong, [1]A.Z. Guo and [2]L.G. Yang
[1]Key Laboratory of Agricultural Animal Genetics,
Breeding and Reproduction of Ministry of Education,
College of Animal Science and Technology,
[2]State Key Laboratory of Agricultural Microbiology,
College of Veterinary Medicine, Huazhong Agricultural University,
430070 Wuhan, P.R. China

Abstract: To investigate the effect of *Helicobacter pylori* on apoptosis of the GES-1 transfected pEGFP-N1-UreB eukaryotic expression plasmid *in vitro*. The *Hp-UreB* gene was amplified by PCR and cloned into eukaryotic expression plasmid pEGFP-NI to construct recombinant plasmid pEGFP-NI-UreB and transfected into GES-1 cells and screened by G418. GES-1 cells transfected into plasmid pEGFP-NI-UreB were co-cultured with *H. pylori* SS1 and its apoptosis was quantified by flow cytometry using Annexin V-APC and 7-AAD. Changes in cell morphology were observed by transmission electron microscope. The recombinant pEGFP-N1-UreB was constructed and stable transfected into GES-1 cells. The apoptotic rates of cells transfected into pEGFP-N1-UreB plasmids were significantly lower than those of control cells ($p<0.05$).

Key words: pEGFP-N1-UreB, *H. pylori* SS1, GES-1, apoptosis, China

INTRODUCTION

Cell apoptosis and proliferation in dynamic balance is importance to maintenance the gastrointestinal tract mucosal tissue integrity. *Helicobacter pylori* (*H. pylori*) is a gram-negative bacterium, specialized in the colonization of the stomach (Warren and Marshall, 1983) and well known as the major gastro-duodenal pathogen of peptic ulcer disease, mucosa-associated lymphoid tissue lymphoma and gastric cancer (Graham *et al*., 1992; Asaka *et al*., 1994; Blaser *et al*., 1995; Delchier *et al*., 2001). In 1994, the World Health Organization International Agency for Research on Cancer (IARC) classified *H. pylori* as a type I (Peter and Beglinger, 2007). However, the mechanism which *H. pylori* can cause ulcers and stomach cancer is still not been well clarified. In recent years some studies have found that the apoptosis rate of the gastric mucosa epithelial cell with *Helicobacter pylori* infection was increased which may be associated with the progression of gastric atrophy (Dooley *et al*., 1989; The EUROGAST Study Group, 1993; Moss *et al*., 1999). Studying on the mechanism of *Helicobacter pylori* induce gastric epithelial cell apoptosis has important significance to further understand the *Helicobacter pylori* virulence carcinogenic mechanism.

Several *H. pylori* virulence factors have been proposed with regard to apoptosis of surface mucous cells and the gastric glands cells including the urease the Vacuolating Cytotoxin (VacA) and the Cytotoxin-Associated gene *A* antigen (CagA) (Covacci *et al*., 1993; Cover and Blaser, 1992; Cover 1996; Hirai *et al*., 1994; Tee *et al*., 1995).

An essential factor in *H. pylori* colonization of the gastric mucosa which also considered a major virulence factor is urease (Eaton *et al*., 1991; Eaton and Krakowka, 1994). Urease is perhaps the most abundant protein made by *H. pylori*, comprising 10% of the total bacterial protein (Dunn and Phadnis, 1998; Evans and Evans, 2000). *H. pylori* possess strong urease activity and produces high concentrations of ammonia which was the production of hydrolysis of urea (Marshall and Langton, 1986; Graham *et al*., 1992). Urease activity of *H. pylori* could produce ammonia which was the major factor for *H. pylori*-induced apoptosis. Ammonia can buffer the pH of its immediate surrounding within the stomach and facilitate nitrogen metabolism of the organism at neutral pH as well as protect it from acid damage at low pH. *H. pylori*-derived ammonia causes injury in isolated human gastric epithelial cells in vitr. Ammonia at concentrations detected in gastric juice in *H. pylori*-infected subjects, causes gastric mucosal injury

Corresponding Author: Liguo Yang, State Key Laboratory of Agricultural Microbiology,
College of Animal Science and Veterinary Medicine, Huazhong Agricultural University,
430070 Wuhan, Hubei P.R. China

(Graham *et al*., 1992; Murakami *et al*., 1993; Takeuchi *et al*., 1995) retards gastric mucosal restitution (Suzuki *et al*., 2000) and induces apoptosis of gastric epithelial cells (Hagen *et al*., 1997). The mechanism by which the urease induces apoptosis of gastric epithelial cells remains unclear however.

This experiment will build eukaryotic expression plasmid and transfected into GES-1 to obtained positive cells by G418 screening to observe the GES-1 cells apoptosis which treated by *H. pylori* SS1 using flow cytometry and transmission electron microscopic technique.

MATERIALS AND METHODS

An Annexin V apoptosis detection kit was purchased from KeyGEN, Nanjing. *H. pylori* Sydney Strain 1 (SS1) was kindly provided by professor Xie Y (Nanchang University, Jiangxi, China). Human gastric epithelial cell line GES-1 was purchased from Xiangya hospital Changsa China. Eukaryote expression vector plasmid pEGFP-N1 was offered from Dr. Guo AZ. All other reagents were obtained from Sigma-Aldrich (Shanghai China).

Construction of UreB antigens eukaryotic vector carrying fluorescent: A pair of primer termed FP and RP was designed as follows: according to *UreB* gene a of *Helicobacter pylori* SS1 in genbank AF508016. FP: 5' AAgCTT gCAT gAC TAA CgA AAC CATA TTC -3'; RP: 5'-CgA ATTCCAA Ag gAT TCA TCA AAC ACg-3'.

HindIII and EcoRI reconition sites were introduced into the 5'-terminal of FP (underlined) and RP (underlined), respectively to facilitate cloning. The Polymerase Chain Reaction (PCR) was done under the following conditions: preheating at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 50 sec, annealing at 55°C for 40 sec and extension at 72°C for 1.5 min. The PCR products were analyzed by agarose gel electrophoresis and recovered using a TaKaRa agarose gel DNA purification kit (China). The fragments were then ligated into the TaKaRa pMD18-T simple vector and sequenced at the Nucleic Acids Facility (Sangon, Shanghai and China). The obtained plasmid was named pMD18-T-UreB and the plasmid was transformed into competent cells *E. coli* DH5a.

The plasmid pMD18-T-UreB was digested with HindIII and EcoRI to obtain the UreB fragment. The purified UreB fragment was ligated into the HindIII-EcoRI digested pEGFP-N1 eukaryote expression vector resulting in pEGFP-N1-UreB and then transformed into competent cells DH5a and then make western blot analysis. The pEGFP-N1-UreB plasmids were extracted and purified from *E. coli* DH5a and waiting to be transfected into GES-1 cells.

Preparation of bacteria *H. pylori* SS1: *H. pylori* Sydney Strain 1 (SS1) was kindly provided by Professor Xie Y (Nanchang University, Jiangxi, China). The *H. pylori* SS1 was cultured at 37°C under microaerobic conditions on Columbia agar plates containing 10% sheep blood. After cultivation for 2 days, the live bacteria were washed by Phosphate-Buffered Saline (PBS, pH 7.4) and centrifuged three times at 5000×g for 15 min.

Transfection of pEGFP-N1-UreB into GES-1 cells: The GES-1 cells were inoculated into 6-well plates at a density of 2×10^5 cells mL^{-1} which be 90-95% confluent at the time transfection. A total of 4 μg purified pEGFP-N1-UreB plasmid (or pEGFP-N1 plasmid), 250 μL Opti-MEM and 10 μL lipofectamine 2000 was added to the transfected group. The mixture was then discarded after 5 h in culture and DMEM containing 10% FBS was added to the culture for an additional 48 h. After 48 h add 500 μg mL^{-1} G418 3 days and G418 (350 μg mL^{-1}) maintenance for screening in 2 weeks until the untransfected cells died and the transfected cells start to divide. The transfected positive cells were selected by G418.

GES-1 cells culture and treatment with *H. pylori* SS1: The transfected GES-1 cells were grew in DMEM medium supplemented with 10% fetal bovine serum 50IU mL^{-1} penicillin and 50IU mL^{-1} streptomycin and incubated at 37°C in a humidified air containing 5% CO_2. The medium was changed every 2 days. Before infection with *H. pylori* the cells were washed by PBS and resuspended in DMEM medium without the antibiotics and incubated in tissue-culture plates for 30 min at 37°C and then cultured for 3 h in DMEM medium without the antibiotics.

The groups of experiment are the GES-1 cells the GES-1 cells transfected pEGFP-N1 plasmid and the GES-1 cells transfected pEGFP-N1-UreB plasmid. The GES-1 cells and the GES-1 cells transfected pEGFP-N1 plasmid were as the control groups. The GES-1 cells were co-cultured with *H. pylori* at 37°C and 5% CO_2 for 24 h. The *H. pylori* bacteria/cell ratio is 20:1.

Determination of cell apoptosis by flow cytometry: The apoptosis of GES-1 cells were quantified by flow cytometry using FITC-conjugated Annexin V and 7-AAD. The GES-1 cells were harvested at indicated time washed with PBS and then harvested by digestion with trypsin 37°C for 5 min. To discriminate between early apoptosis and necrosis the cells were simultaneously stained with Annexin V-APC and 7AAD and detected with flow cytometry (BD FACSCalibur, USA) according to the manufacturer's instruction (Apoptosis Detection kit, KeyGEN, Nanjing). Experiments were repeated three times independently. At least 10,000 cells were counted in each sample.

Transmission electron microscopic observation: After GES-1 cells treated with *H. pylori* SS1, cells were fixed for electron microscopy with the buffer containing 1.25% glutaraldehyde, 2.5% paraformaldehyde, 0.06% picric acid and 0.06% $CaCl_2$ with 0.1M cacodylate buffer (pH 7.2) for 5 min on ice. They were then postfixed with 1% OsO_4 buffered and 0.1M cacodylate buffer (pH 7.2) for 5 min on ice. After dehydration with a graded series of ethanol the samples were removed from the dishes by propylene oxide and were embedded in TAAB. Thin sections were cutted and stained with 1% uranyl acetate and lead citrate. The sections were observed with a Hitachi H-7100 electron microscope.

Statistics: All analyses were performed using SAS System 8.1 Software. The data of experimentation were compared by a Student's t-test, expressed as mean±SD. The $p<0.05$ were considered to be significant.

RESULTS AND DISCUSSION

Amplification of *UreB* gene by PCR: The target fragment of *UreB* gene with the expected size amplified from DNA template of *H. pylori* SS1 is shown in Fig. 1 (left). The pEGFP-N1-UreB was performed successively, enzyme digestion analysis (right) and sequencing. The nucleotide sequences of *UreB* gene were completely same with the *UreB* gene of *H. pylori* SS1 in genbank AF508016. Proteins were extracted and assayed by Western blotting using monoclonal antiserum to UreB (Santa Cruz Biotechnology). The results indicated that rUreB was produced in the supernatant of pEGFP-N1-UreB (Fig. 2).

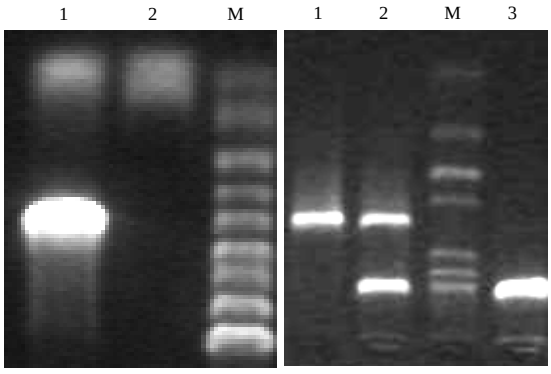

Fig. 1: The target fragment of *UreB* gene amplified from *H. pylori* SS1 DNA by PCR (left) and the transfected pEGFP-N1-UreB plasmids was digested (right). Lane M: 1 kb DNA marker; Lane 1: the target amplification fragment of *UreB* gene (1134 bp); Lane 2: negative control (left). Lane M: 1 kb DNA marker; Lane 3: the pEGFP-N1; Lane 2: the digested pEGFP-N1-UreB; Lane 1: UreB (right)

Cells which transfected with pEGFP-N1-UreB: These GES-1 cells transfected plasmids pEGFP-N1-UreB exhibited bright green fluorescence under fluorescent microscope (Fig. 3). The transfected GES-1 showed an elongated fibroblastic appearance with concomitant EGFP fluorescence under fluorescent microscopy. The GES-1 transfected into pEGFP-N1-UreB (pEGFP-N1) plasmids exhibited bright green fluorescence under fluorescent microscope.

GES-1 cell apoptosis and necrosis induced by *H. pylori*: In this experiment, flow cytometry using FITC-conjugated Annexin V and 7-AAD revealed that GES-1 cells exposed to *H. pylori* underwent apoptosis and

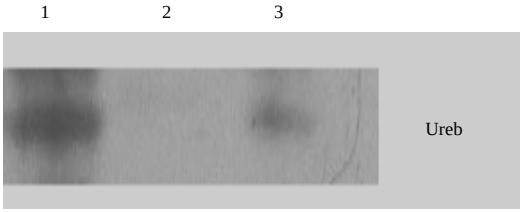

Fig. 2: Western blotting analysis of UreB in transgenic bacterium. Lane 1: Western blotting of DH5a with pEGFP-N1-UreB; Lane 2: Western blotting band of DH5a; Lane 3: Western blotting band of DH5a with pEGFP-N1-UreB

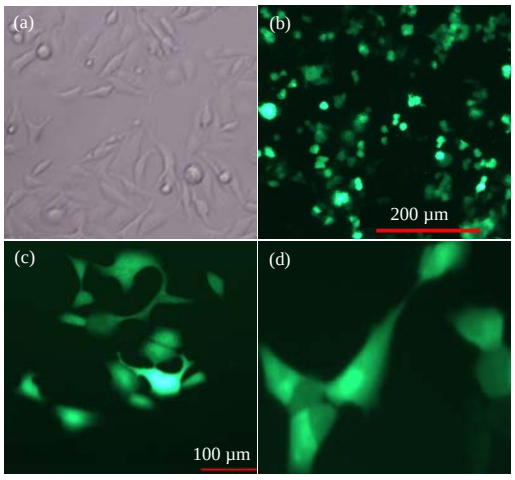

Fig. 3: The GES-1 transfected into pEGFP-N1-UreB plasmids. a) The GES-1 cells before transfected (200x); b) The GES-1 cells after transfected (100x); c) The GES-1 cells after transfected (200x) and d) The GES-1 cells after transfected (400x)

necrosis. Results showed that the apoptotic rates of GES-1 cells transfected into pEGFP-N1-UreB plasmids were significantly lower than those of control cells ($p<0.05$) (Fig. 4).

Transmission electron microscopic observation: Before treatment with *H. pylori* SS1, GES-1 cells grow closely, spindle-shaped, polygonal and rarely deciduous. But after treatment, the cells gradually into a sphere shape, cell volume also gradually become smaller resulting in cell spacing increases, cells exfoliated increased. With 0.25% neutral glutaraldehyde fixation, observation with transmission electron microscopic revealed that part of the cells were morphologically apoptosis. The nucleus chromatin agglutinated irregularly, forming different sizes clumps cytoplasm has multiple vacuoles some visible intact organelles and apoptotic bodies exited in the cytoplasm.

Under TEM, the volume of necrotic cell is reduced, the nucleoplasm ratio increases, the nucleoplasm closely, cell surface microvilli disappeared, cell membrane collapse (Fig. 5).

The infection of *H. pylori* is deeply associated with the development of peptic ulcer disease and cancer in the host stomach. The gastric mucosal epithelial cells *in vitro* co-cultured with *Helicobacter pylori* can cause gastric mucosa epithelial cell apoptosis (Chen *et al*., 1997; Nagata, 1997; Fan *et al*., 1998; Yang *et al*., 2003; Zong *et al*., 2005; Xu *et al*., 2009). Animal experiments also found that the gastric mucosa epithelial cell apoptosis rate increased when *Helicobacter pylori* infected and after eradication of *Helicobacter pylori* the apoptosis rate decreased significantly (Jones and Sherman, 1999). Kohda *et al*. (1999) also found that *H. pylori* infection can induce gastric mucosa cell apoptosis. When the *Helicobacter pylori* were eradicated after treatment the apoptosis rate began to fall. However, the apoptosis rate is still higher than that the control group in 3 months.

This report shows that under G418 (500 μg mL^{-1}) culture and G418 (300 μg mL^{-1}) maintenance for screening in 2 weeks, several positive cell clones survived. The results of RT-PCR and agar diffusion reaction indicated

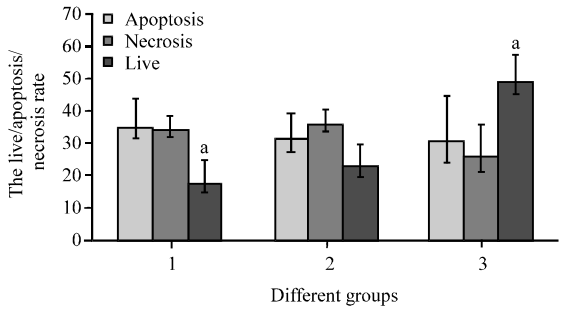

Fig. 4: The live/apoptosis/necrosis rate of different GES-1 cells using. Annexin V-APC/7-AAD (Means±SD) ($^{a}p<0.05$)

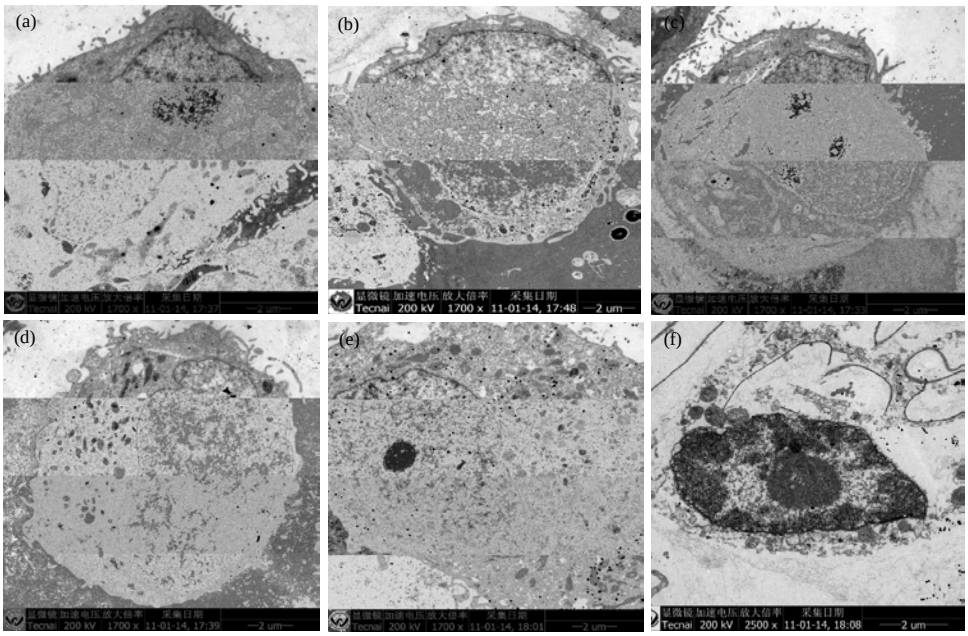

Fig. 5: The ultrastructural photographof GES-1 cells after 48 h incubation with *H. pylori*: showing apoptosis and necrosis. a-d) Apoptotic cells by transmission electron microscopy (1700x); e and f) Necrotic cell transmission electron microscopy (1700x)

that the *UreB* gene and had been highly expressed in GES-1 cells. It is a highly effective method to screen the transfected cells using G418.

In this experiment, researchers chose the vector pEGFP-N1 which inserted by UreB to expression in GES-1 cells. It proved that the pEGFP-N1 is suitable for gene transferred into eukaryotic cell which is safe and effective. The infection of *H. pylori* can induce apoptosis of gastric epithelial cells which may increase the risk of the development of gastric cancer.

The investigation of the GES-1 cells death triggered by *H. pylori* indicates a role of *H. pylori* in the induction of apoptosis. Researchers also showed that plasmid pEGFP-N1-UreB can decelerate GES-1 cells apoptosis when co-culture with *H. pylori*. The GES-1 cell line in this experiment used is from normal fetal gastric mucosa epithelial cells and no tumor formation. It was found that the apoptosis rate and mortality of GES-1 cells transfected pEGFP-N1-UreB less than the cells which no transfected plasmid and transfected pEGFP-N1 plasmid. The study of Yang *et al*. (2003) also found that the apoptosis rate of epithelial cell was increasingalong with the concentration of *Helicobacter pylori*.

Although, the apoptosis rate and death rate of GES-1 cells which transfected plasmid pEGFP-N1-UreB death rate less than those of the control group but the GES-1 cells coexisted with *Helicobacter pylori* for a long time were dead or apoptosis. Electron microscopy also revealed that the morphology of necrotic cell was destroyed. Membrane and nuclear was meltdowned the cell contents was leaked; organelles was disappeared and the fluff was disappeared or became short. The apoptotic cells showed the nucleoplasm agglutinated to form different sizes clumps; cytoplasm has many vacuoles and the apoptotic bodies existed in the cytoplasm simultaneously.

About the apoptosis mechanism of helicobacter pylori induced gastric mucosal cell someone think it was caused by *H. pylori* urease. The research results of Tsuji *et al*. (1993) showed that the ammonia which urea decomposited products can pass through the cell membrane, damage organelles and induce apoptosis resulting in gastric mucosal cell shedding, mucosal atrophy. Also, other one thinks that the mechanism of apoptosis is *Helicobacter pylori* activated neutrophils to produce large amounts of reactive oxygen metabolites. The experiments of Naito *et al*. (1997) showed that the inhibition to gastric epithelial cells was stronger along with the NH_4Cl concentration which can induce the apoptosis. The findings of Piotrowski *et al*. (1997) showed that the *H. pylori* UreB protein not only induced gastric mucosal acute inflammatory response but also leads to the apoptosis rate of mucosal epithelial cells and glandular cell increased. The above results showed the *H. pylori* UreB protein can promote gastric epithelial cell apoptosis. The above results are described of *Helicobacter pylori* UreB protein in inducing cell apoptosis plays an important role. If *UreB* gene was transfected into the epithelial cells, there may be inhibited the cell apoptosis. The results of the experiment also found that the apoptosis rate of GES-1 cells which transfected into plasmid pEGFP-N1-UreB was less than that of the control group. But long time and large dose of *Helicobacter pylori* treatment transfected cells can also lead to apoptosis and necrosis. The effect of apoptosis and necrosis may be have other way in addition to urease.

CONCLUSION

GES-1 cells which transfected into pEGFP-N1-UreB plasmids can block *H. pylori* infection and apoptosis.

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