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Short communication

Early apoptosis of monocytes induced by *Helicobacter pylori* infection through multiple pathways

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A B S T R A C T

Only a small percentage of people infected with *Helicobacter pylori* (*H. pylori*) will develop overt chronic gastric diseases. To understand the pathological mechanism, the action of *H. pylori* on monocyte apoptosis was detected. *H. pylori* co-culturing with peripheral blood monocytes, THP-1 or U937 cells result in early apoptosis at 6, 12, and 24 h after infection. The phosphorylated Bad and JNK were increased, and Bcl-2 was declined at 6, 12, and 24 h in peripheral blood monocytes after *H. pylori* infection. The phosphorylated Akt was augmented at 6 and 12 h post-infection. A slow apoptotic response was induced by *H. pylori* via Bad and Bcl-2 regulators, activated caspase-8 and caspase-9, and JNK at 24 h in THP-1 cells. Meanwhile, only Bad and JNK were involved in regulating U937 cells apoptosis at 24 h after infection. These results supported a novel mechanism of *H. pylori* escaping from monocytes by upregulation of early apoptosis and inhibition of late apoptosis. The differences among the three cells may reveal why *H. pylori*-derived disease occurs in relatively few people and provide a pathological mechanism whereby a treatment for *H. pylori*-derived disease may be developed.

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1. Introduction

Helicobacter pylori (*H. pylori*) is a Gram-negative bacterium colonizing the epithelial layer of the human stomach (Tohidpour, 2016; Bücker et al., 2012). According to current available data, approximately 50%–80% of the world's population is infected with *H. pylori*, but only a relatively small percentage of individuals will develop overt chronic gastric diseases (Chomvarin et al., 2012). *H. pylori* has co-evolved in the gastric micro-environment and gastric epithelial cells of humans for over 58,000 years, hence most humans present with no clinical symptoms nor chronic *H. pylori* infection (Otero et al., 2014).

H. pylori may evade its host's immune system and present long-term symptoms in affected individuals. While it can stimulate both innate and acquired immune responses, *H. pylori* may down-regulate the host's immune response to avoid its own clearance and thus maintain a relatively peaceful co-existence within the host (Robinson et al., 2007). In fact, the host's innate immune response plays a central role in allowing *H. pylori* to escape the host's immunoreaction (Yuan et al., 2009). Pathological changes in the gastric epithelium of the stomach after infection by *H. pylori* have been researched for decades (Smith, 2014; Alzahrani et al., 2014). After *H. pylori* infection, gastric epithelial cell changes involve epithelial junctions, DNA damage, apoptosis, proliferation, stimulation of cytokine production, and cell transformation (Alzahrani et al., 2014). *H. pylori* infection induces cytokine and chemokines that are secreted from gastric epithelium cells, which can lead to the recruitment of monocytes/macrophages to the gastric mucosa (Smith, 2014).

Apoptosis is defined as an active physiological process of cellular self-destruction. It plays an essential role in maintaining cellular homeostasis during removal of invading microbial pathogens and clearance of dying cells (Sun and Shi, 2001; Ashktorab et al., 2008). During pathological changes, there are early apoptosis and late apoptosis stages. In early apoptosis, the integrity of the plasma membrane of gastric cells is maintained, however phosphatidylserine (PS) appears on the cell surface, which can be recognized by phagocytes. Late apoptosis of the plasma membrane is characterized by permeabilization (Gregory and Devitt, 2004).

H. pylori can partially evade monocytes-mediated phagocytosis, which is as an important mechanism of the innate immune defense system (Robinson et al., 2007; Yuan et al., 2009). The innate immune system has evolved to allow multicellular organisms to survive with microbes that can cause inflammation and damage to the

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host tissue (Auffray et al., 2009). *H. pylori* has evolved to influence the innate immune system to allow long-term infection. This represents a key factor in the etiology of various gastroduodenal diseases, ranging from chronic gastritis to the development of peptic ulcer disease and gastric cancer (GC) (Chomvarin et al., 2012; Zhang et al., 2016).

Monocytes are the first immune defense line in humans and they play important roles in the inflammatory response to the pathogens (Auffray et al., 2009). Monocytes migrate from the bloodstream to target tissues and differentiate into tissue-resident macrophages or dendritic cells, which play an important role in eliminating dying (apoptotic) cells through phagocytosis. The apoptosis of monocytes is an important mechanism in immune regulation, as it maintains homeostasis in the immune system. Defective apoptotic cell clearance will result in an immune tolerance, such as autoimmune disease (Srivastava et al., 2010). Therefore, determining the effects of *H. pylori* on monocyte apoptosis and the molecular regulation network will allow us to better understand *H. pylori*-derived disease. Moreover, it will be useful for understanding the susceptibility of some people to *H. pylori* infection, which importantly can lead to chronic *H. pylori* infection in those individuals.

In this study, we focused on the apoptosis mechanism induced by *H. pylori* in monocytes. We initially established a *H. pylori* infection model in three monocyte cells: peripheral blood monocytes, THP-1 and U937. Then we analyzed the status of the apoptosis and the activities of some pivotal apoptotic proteins at different times in this model. These results provide some interesting new data that may attract researchers focused on *H. pylori*-generated apoptotic monocytes involved in immune tolerance or chronic *H. pylori* infection.

2. Materials and methods

2.1. *H. pylori*

The *H. pylori* international standard strain 26695 was provided by the *H. pylori* Research Laboratory of the Chinese Center for Disease Control and Prevention (Beijing, China). *H. pylori* 26695 was grown on chocolate agar plates supplemented with 10% sheep's blood at 37 °C under microaerophilic conditions (5% O_2, 10% CO_2, and 85% N_2) (Gong et al., 2015).

2.2. Monocytes

The human peripheral blood monocytes were obtained from Otwo Biological Technology Co., Ltd. (Guangzhou, China), and THP-1 and U937 cell lines were obtained from SGST, Institute of Biochemistry and Cell Biology, SIBS, and CAS (Shanghai, China). They were maintained in RPMI 1640 medium, supplemented with 10% fetal bovine serum (FBS) under a humidified atmosphere of 5% CO_2 at 37 °C. The cells were harvested once they reached the logarithmic growth phase.

2.3. Co-culture of monocytes with *H. pylori*

H. pylori were harvested in chocolate agar broth and quantified by a spectrophotometer reaching 1×10^8 CFU/ml. Bacteria were added into the cells at a multiplicity of infection (MOI) of 100:1 and co-cultured for 0, 6, 12, and 24 h. Non-*H. pylori*-containing cells (peripheral blood monocytes, THP-1 or U937) were used as negative control groups. The cells were then harvested at the indicated time for cellular apoptosis detection and analysis of the apoptosis-related factors.

2.4. Cellular apoptosis assays by flow cytometry

Cellular apoptosis assay of peripheral blood monocytes, THP-1 and U937 cells were performed by using an Annexin V-FITC Apoptosis Detection Kit (BD, Franklin Lakes, NJ) according to the manufacturer's instructions. Briefly, 1×10^5 cells were mixed together with 500 μl of Annexin Binding Buffer, 5 μl of FITC-conjugated Annexin V antibody, and 5 μl of propidium iodide (PI), respectively. The mixture was incubated for 15 min in a dark chamber at room temperature. The relative number of apoptotic cells was determined using a Beckman Coulter EPICX-XL Flow Cytometer (Beckman Coulter, Inc., USA). The normal cells without induction of apoptosis were used for fluorescence compensation regulation to remove the spectral overlap.

2.5. Measurement of the activated (phosphorylated or cleaved) Bad, Bcl-2, caspase-8, caspase-9, Akt, and JNK proteins by Luminex xMAP® technology

The activated (phosphorylated or cleaved) Bad, Bcl-2, caspase-8, caspase-9, Akt and JNK proteins were analyzed based on Luminex xMAP® technology-consisting of a fluorescent-coded magnetic bead with specific antibody combined with biotinylated antibody and a streptavidin conjugate reporting system (Lam and Roberts-Rapp, 2014). The detected phosphorylated sites for each protein are: Ser112 of Bad, Ser70 of Bcl-2, and Ser473 of Akt, respectively. The activated caspases were identified at the cleaving site of Asp384 for active caspase-8 and Asp15 for active caspase-9. The assay process was based on the product menu and instructions of the Early Apoptosis 7-plex Magnetic Bead Kit (Millipore, Billerica, MA).

Briefly, the cells were lysed with MILLIPLEX® MAP Lysis Buffer containing protease inhibitors. A total of 20 μg protein from each lysate was diluted in MILLIPLEX® MAP Assay Buffer at 4 °C overnight. The Median Fluorescence Intensity (MFI) was measured with the Luminex 100/200 system (Luminex, Madison, WI). The relative activities of Bad (Ser112), Bcl-2 (Ser70), active caspase-8 (Asp384), active caspase-9 (Asp15), Akt (Ser473), and JNK (Thr183) proteins were converted to fluorescence intensity (FI) as follows: FI of Bad, Bcl-2, caspase-8, caspase-9, Akt, and JNK/FI of Tublin × 5000.

2.6. Statistical analysis

All data were subjected to one-way analysis of variance (ANOVA). Differences were considered to be statistically significant at a p value of 0.05 or less.

3. Results

3.1. Apoptosis in *H. pylori*-infected peripheral blood monocytes, THP-1, and U937 cells

The early apoptosis rates in peripheral blood monocytes, THP-1, and U937 cells were increased at 6, 12, and 24 h after *H. pylori* infection compared to the control group. The late apoptosis rates in peripheral blood monocytes were only up-regulated at 12 h post-*H. pylori* infection, which were increased at 12 and 24 h in U937 cells treated by *H. pylori*. No substantial changes in late apoptosis rates of THP-1 cells were observed after infection with *H. pylori* during the experiments (Fig. 1).

In peripheral blood monocytes cells, there were early apoptotic rates of 78.03 ± 4.03%, 62.03 ± 8.34% and 43.60 ± 3.02% at 6, 12 and 24 h post *H. pylori* infection, respectively (Fig. 1a), which were 25.36-fold (two sample t-test, $p = 0.001$), 20.16-fold (two sample t-test, $p = 0.010$) and 14.17-fold (two sample t-test, $p = 0.003$)

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