財團法人明日醫學基金會專題研究計畫申請書

一、基本資料： 申請條碼：

本申請案所需經費(單選)	■A 類(執行計畫所需經費) ☐B 類(研究主持費，限人文處計畫，不須填寫表 C002 及 C004 至 C009)				
計畫類別(單選)	■一般型研究計畫 ☐特約研究計畫 ☐新進人員研究計畫 ☐其他				
研究型別	■個別型計畫 ☐整合型計畫				
申請機構/系所(單位)	長庚大學醫學院微生物及免疫學科				
本計畫主持人姓名	賴志河	職稱	教授	身分證號碼	
本計畫名稱 中文	探討 HMGB1 參與幽門螺旋桿菌誘導胃上皮細胞發炎之機制				
本計畫名稱 英文	The involvement HMGB1 in *Helicobacter pylori*-induced inflammation in gastric epithelial cells				
整合型總計畫名稱					
整合型總計畫主持人				身分證號碼	
全程執行期限	自民國 113 年 1 月 1 日起至民國 113 年 12 月 31 日				
研究學門(請參考本申請書所附之學門專長分類表填寫)	學門代碼	名稱(如為其他類，請自行填寫學門)			
研究性質	■純基礎研究 ☐導向性基礎研究 ☐應用研究 ☐技術發展				
本計畫是否為國際合作計畫	■否； ☐是，合作國家：_____，請加填表 I001～I003				
本計畫是否申請海洋研究船	■否； ☐是，請務必填寫表 C014。				
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三、主要研究人力：

（一）請依照「主持人」、「共同主持人」、「協同研究人員」及「博士後研究」等類別之順序分別填寫。

類 別	姓名	服務機構/系所	職稱	在本研究計畫內擔任之具體工作性質、項目及範圍	*每週平均投入工作時數比率(%)
主持人	賴志河	長庚大學/醫學院/微生物及免疫學科	教授	研究之規劃及推動、實驗設計、整理數據、撰寫研究成果與發表論文	60%

※註：每週平均投入工作時數比率係填寫每人每週平均投入本計畫工作時數佔其每週全部工作時間之比率，以百分比表示（例如：50%即表示該研究人員每週投入本計畫研究工作之時數佔其每週全部工時之百分五十）。

Abstract

Helicobacter pylori has been recognized as an important pathogen that was found in patients with peptic ulcer and gastric adenocarcinoma. *H. pylori* cytotoxin-associated gene A (CagA) has been demonstrated that associated with higher levels of interleukin-8 (IL-8) production in gastric epithelial cells. High mobility group box-1 (HMGB1) is an inflammation-related cytokine that binds with receptors for advanced glycation end-products (RAGE), which trigger the inflammatory response. It has been reported that *H. pylori* infection not only induces autophagy but also inhibits lysosome fusion with autophagosome. However, the molecular mechanism of how *H. pylori* CagA triggers HMGB1, as well as the pathway between HMGB1 and autophagy, have not been extensively studied. In this study, we will investigate whether CagA influences HMGB1 expression in gastric epithelial cells. Furthermore, we will explore the correlation between HMGB1 and autophagy, which is involved in *H. pylori* CagA-induced inflammation. The results from this study will provide an insightful understanding of how *H. pylori* utilizes its virulent factor to trigger inflammation formation.

Keywords: *Helicobacter pylori*; cytotoxin-associated gene A; HMGB1; inflammation

A. Introduction

Helicobacter pylori, a gram-negative bacterium, grows in microaerophilic conditions. The primary reservoirs of *H. pylori* are human and usually cause gastritis, peptic ulcers, and gastric adenocarcinoma (1). Since the antibiotic resistance of *H. pylori* is rising, which has made antibiotic treatment ineffective recently, multiple treatment regimens are in use (2).

High mobility group box-1 (HMGB1) is a ubiquitous nuclear DNA-binding protein that stabilizes nucleosome, enables nicking of DNA, and facilitates transcription (3). It is not only a newly recognized factor that can regulate cancer cell tumorigenesis, expansion, and invasion but also serves as a cytokine that mediates late lethal systemic inflammation via its translocation from nuclear to cytosol (4). Receptor for advanced glycation end-products (RAGE), one of receptors for HMGB1, which can amplify the proinflammatory signaling(5). Upon HMGB1 interacts with RAGE, which triggers the inflammatory response including mitogen-activated protein kinases (MAPKs) actives nuclear factor (NF)-κB, and subsequently enhances the production of interleukin 8 (IL-8) (5).

Autophagy is defined as a degradation pathway in which cytoplasmic content is engulfed and degraded by the lysosomes. Autophagy plays an important role in eliminating extra proteins, damaged organelles, and intracellular microbes, which regulates cellular homeostasis(6). *H. pylori* infection induces autophagic vesicle formation, which is associated with the multiplication of the *H. pylori* in the infected macrophages (7). There are two virulence factors are found contributing to it, including cytotoxin-associated gene A (CagA) and vacuolating cytotoxin A (VacA). Both of them are localized in lipid raft microdomains, and co-effected to induce autophagy of *H. pylori*-infected cells. VacA can induce cell vacuolation and its channel-forming activity is important for inducing autophagy (8). CagA translocates into host cell membrane via a type IV secretion system (TFSS) and then induces inflammation (9). However, the association of CagA and HMGB1, as well as the signaling activation between HMGB1 and autophagy are not well investigated yet. In the present study, the specific aims of this proposal that we intent to perform are:

1. Investigating whether CagA influences translocation of HMGB1 in the gastric epithelial cells.
2. Exploring the order between HMGB1 and autophagy that involved in *H. pylori* CagA functions.
3. Studying the roles of autophagy in enhancing *H. pylori*-infected cell survival to promote inflammation.

B. Preliminary results

For the purpose of investigating the autophagy pathway involved in *H. pylori* infection, we established an *in vitro* cell-based experimental platform. As shown in Fig. 1A, wildtype *H. pylori*-induced LC3-II/I conversion was increased with the raising MOI but decreased in the MOI of 200. Thus, we selected the MOI = 100 as *H. pylori* infection condition and performed a time course analysis. The results showed that an increase level in LC3-II/I conversion is associated with the time of *H. pylori* infection (Fig. 1B). These results indicate that *H. pylori* induces autophagy activation in AGS cells, and that the optimal conditions for infection are an MOI of 100 and incubation for 16-24 h.

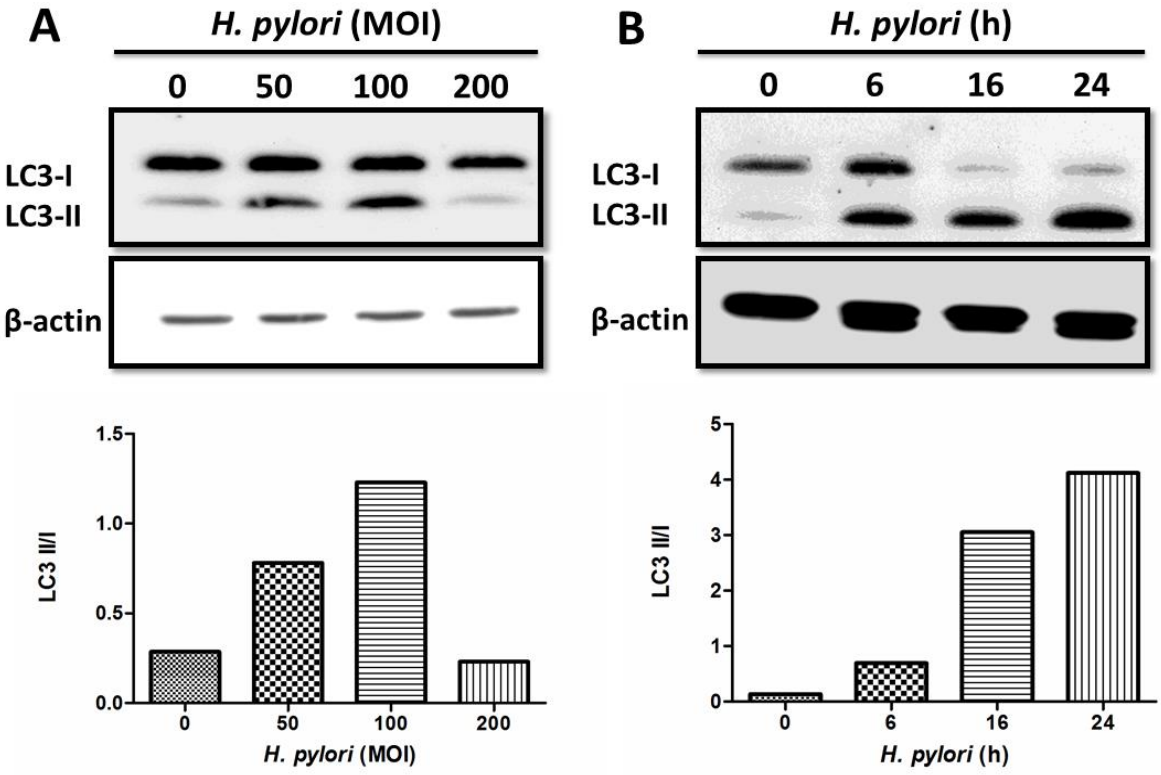

Figure 1. Wildtype *H. pylori* (VacA+CagA+) infection induces autophagy activation. (A) Cells were incubated with *H. pylori* at different MOIs (0-200) for 6 h. (B) Cells were incubated with various times (0-24h) at MOI 100 of *H. pylori*. Western blot analysis was performed to determine the protein expression levels of LC3-II/I (lower panel). β-actin was used as the loading control.

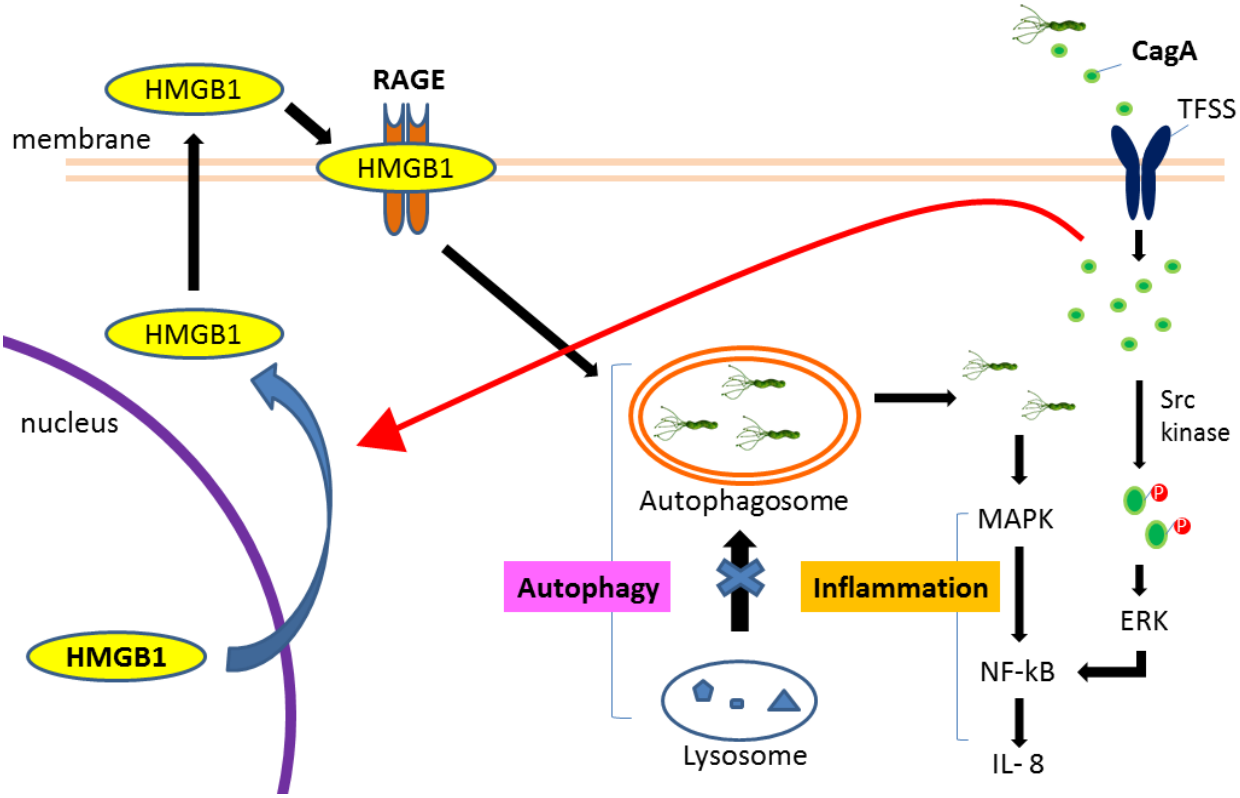

Figure 2. CagA induces HMGB1 translocation to enhance autophagy and downstream reaction. *H. pylori* infection enhances HMGB1 and RAGE expression. Binding of those two molecules that triggers the autophagy machinery to enhance the *H. pylori* survival flows by activation of inflammation pathway. The virulence factor CagA translocates into the host cells via type four secretion system (TFSS), and then phosphorylated by Src kinase; subsequently triggers the ERK that increases the inflammation formation. However, the role of CagA in the HMGB-induced autophagy and inflammation pathway remains unknown.

C. Materials and methods

1. Culture of *H. pylori*

To establish an assay platform for *H. pylori*-infected gastric epithelial cell, wildtype ($VacA^+CagA^+$) *H. pylori* 26695 (ATCC 700392) and CagA-knockout strain ($CagA^-$) will be cultured and incubated on 10% sheep blood agar plates in a microaerophilic environment (10% CO_2, 5% O_2, 85% N_2) at 37°C. All experimental strains will be incubated for 24–36 h to achieve optimum microbial activity as described previously (10).

2. Culture of gastric epithelial cells

Human gastric epithelial cells (AGS cells, ATCC CRL 1739) will be cultured in F12 (GibcoBRL). Ten percent de-complemented fetal bovine serum (Hyclone) will be added to all cultures. Penicillin and streptomycin (GibcoBRL) will be used if necessary. Antibiotics will not to be added to the cell culture medium in the *H. pylori*-infected assay.

3. Infection of cells with *H. pylori*

AGS cells (1×10^6) will be seeded in 6-well plates and incubated at 37 °C for overnight. *H. pylori* will be suspended in 37°C pre-warm culture medium contained 10% FBS, and the bacterial suspensions will be adjusted to 1×10^9 CFU/ml. The cells will then be infected with *H. pylori* at a multiplicity of infection (MOI) of 100 at 37°C for an additional 16 h .

4. Knocking down HMGB1 with siRNA

The RNA interference experiment will be performed using the Silencer Select Pre-designed siRNA (Invitrogen) according to the manufactur's instructions. AGS cells will be transfected with siRNA. Twelve hours after the transfection, the conditioned media will be replaced with DMEM containing 10% FBS. After transfection for 24 h, the conditioned media will be replaced completed medium..

5. Quantitative real-time reverse transcription-PCR

HMGB1 mRNA will be isolated, and cDNA will be synthesized by PrimeScript RT reagent Kit (Takara, Shiga, Japan). RT-PCR will be carried out in a Lightcycler (Roche, Basel, Switzerland). The primers will be used are corresponded to human HMGB1 (forward, 5′-AGGATCCCAATGCACCCAAG-3′ and reverse, 5′-CGCAACATCACCAAT GGACAG-3′). The program will be pre-incubated at 50°C for 2 min and 95°C for 10 min; PCR will be performed with 40 cycles of 95°C for 10 s and 60°C for 1 min.

6. Gentamicin protection assay

AGS cells will infect with *H. pylori* (MOI = 100) for 16 h. The *H. pylori*-infected cells will be washed with PBS three times and then incubated in sterile water at 37°C for 10 min to osmotically disrupt the cell membrane. The resulting lysates will be then diluted in PBS and applied to Brucella blood agar plates. Viable *H. pylori* colonies will be enumerated after 3–5 days of incubation and the intracelllular survival rates will be presented as colony-forming units (CFU).

7. Western blot analysis

H. pylori-infected AGS cells will be washed three times with PBS and then boiled in

SDS-PAGE sample buffer for 10 min. The samples will be resolved by 10-12% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore). The membranes will be incubated with primary antibodies specific against autophagy-associated proteins (i.e. LC3-I, LC3-II, beclin-1, and p62) at room temperature for 1 h. The blots will be washed and then incubated with horseradish peroxidase-conjugated secondary antibody (Millipore). The proteins of interests will be detected using ECL Western blotting detection reagents (GE Healthcare) and will be visualized using X-ray film (Kodak) (11).

8. Statistical analysis

Experimental results will be expressed as the mean ± standard deviation of independent triplicate experiments. Student's *t*-test wwill be used to calculate the statistical significance of differences between the two groups. Differences wwill be considered significant at $P < 0.05$. Statistical analysis wwill be performed using Prism6 (Graph Pad, San Diego, CA, USA).

D. Anticipated results

1. *H. pylori* CagA induces HMGB1 expression to enhance autophagy

To carry out this study, we will investigate the order between HMGB1 expression and autophagy activation in *H. pylori* CagA-infected cells. The virulence factor, CagA, can translocate into cytoplasm by associated with the membrane-receptor integrin, and its delivery is cholesterol-dependent (12). Additonally, RAGE, the receptor for HMGB1, is associated with glycation end-product. Thus, we propose that the *H. pylori* CagA may induce HMGB1 expression and then enhance the autophagy pathway. If this is the case, knocking down HMGB1 may inhibit the activation of autophagy.

2. Autophagy enhances the *H. pylori* survival in AGS cells.

It has been reported that autophagy is a lysosome-mediated, self-degradation process that protects normal cells, but also promotes tumor cell survival under stress (13). Since autophagy can play a role of survival signal (14), we then suggests that autophagy may enhance the survival of *H. pylori* in gastric epithelial cells. To demonstrate this hypothesis, we will perform gentamicin protection assay to analyze the *H. pylori* intracellular survival status, and the higher survial rate should be observed.

3. *H. pylori* CagA plays an important role in the regulation of HMGB1-induced inflammasion.

Autophagy which induced by HMGB1 is asscoiated with cell survival mechanism that reported in several studies recently (15). Accordingly, we propose that wildtype ($CagA^+$) *H. pylori* infection obtains higher survial rate in human gastric epithelial cells than that of mutant ($CagA^-$) strain. As the higher *H. pylori* survial, the higher inflammation should be induced. If this is in case, an increase of inflammasion formation in $CagA^+$ *H. pylori*-infected gastric epithelial cells should be observed in this study.

F. References

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