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本年度申請主持國科會各類研究計畫(含預核案)共___件。(共同主持之計畫不予計入) 本件在本年度所申請之計畫中優先順序(不得重複)為第___。					
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本計畫是否有進行下列實驗：(勾選下列任一項，須附相關實驗之同意文件) □人體實驗/人體檢體 □人類胚胎/人類胚胎幹細胞 ■基因重組實驗/基因轉殖田間試驗 □動物實驗					
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計畫主持人(申請人)簽章：_____ 日期：

Study on Cholesterol glucosylation by cholesterol-α-glucosyltransferase delays phagocytosis of *Helicobacter pylori* by macrophages

胃幽門螺旋菌膽固醇醣化酵素延遲巨噬細胞吞噬作用之研究

中文摘要

胃幽門螺旋菌 (*Helicobacter pylori, H. pylori*) 是微需氧(microaerophilic)的革蘭氏陰性菌，全世界大約有50%的人都有感染，感染胃幽門螺旋菌證實跟許多胃部疾病發生有所關聯（如胃潰瘍、十二指腸潰瘍、慢性胃炎、甚至是胃癌等）。近年來的研究指出：當幽門螺旋菌結合到胃部表皮細胞時，它會將表皮細胞細胞膜上的膽固醇吸取到細菌的體內，而後藉由胃幽門螺旋菌中的膽固醇醣化轉移酵素cholesterol-α-glucosyltranferase (HP0421又稱為CapJ)，將葡萄糖 (glucose) 轉移到外來的膽固醇上，形成醣化膽固醇，並呈現到胃幽門螺旋菌的細胞膜。這些醣化膽固醇被證實與胃幽門螺旋菌逃避宿主免疫反應有極大的相關聯性。同時，先天性的免疫反應如巨噬細胞的吞噬作用，對於*capJ*基因剔除菌株則較易被巨噬細胞吞噬。但是對於巨噬細胞吞噬作用之後細胞內細菌的存活率及相關的先天性免疫反應的影響仍不清楚。在本提案，首先利用gentamicin protection assay方法測定巨噬細胞吞噬作用後細胞內細菌的存活率，發現*capJ*基因剔除菌株無法有效的抵抗巨噬細胞的毒殺反應；同時，藉由cell adhesion assay方法也觀察到*capJ*基因剔除菌株黏附至巨噬細胞的能力也比正常野生菌株(26695)來的低。此結果說明CapJ活性與保護胃幽門螺旋菌免於巨噬細胞毒殺有關；同時也與細菌的黏附力有關。進一步我們將利用溶小體(lysosome)的指示劑，LysoTraker Red與neutral red追蹤巨噬細胞內的細菌。研究正常野生菌株是否可以抵抗吞噬小體與溶小體之融合而*capJ*基因剔除菌株則是否缺非此功能。藉由此研究探討醣化膽固醇在延遲巨噬細胞吞噬作用以逃避免疫反應的過程中所扮演的角色。

Abstract

Helicobacter pylori (*H. pylori*) belongs to spiral Gram-negative bacteria and infecting approximately 50% people around the world. Infection with *H. pylori* is associated

with many gastric diseases including gastric ulcer, duodenal ulcer, and gastric cancer. *H. pylori* extracts cholesterol from host cell membranes and converts it into glucosyled cholesterols by a membrane-bound enzyme cholesterol-α-glucosyltranferase (HP0421, *capJ*). Genetic experiments of mutants which lacking CapJ show that those mutants exhibited a higher level of engulfment by macrophages, suggesting the role of CapJ in bacterial escaping phagocytosis by macrophages. However, the subsequent intracellular survival activity between the wild-type and *capJ* knockout strains has not been demonstrated. In this study, the gentamicin protection assay was utilized to assess the intracellular survival of *H. pylori.* We found that the *capJ* knockout strain had a lower level of intracellular activity as well as reduced adhesion activity in murine macrophages J774.A1 cells, suggesting that CapJ was crucial to protect phagocyted bacteria from intracellular killing. We further compared the level of engulfed bacteria in phagosomes between the wildtype and *capJ* mutant bacteria using an indicator of lysosomes, LysoTraker Red. At post-infection of 120 min, we found a significantly lower level of engulfed bacteria in the phagosomes of the wildtype *H. pylori*. In parallel, the level of neutral red acuminated in the acidic compartments including late endosomes and lysosomes was assessed; we observed that macrophages infected with *capJ* mutants accumulated more neutral red than wild-type *H. pylori* did. These results suggested that CapJ is crucial to facilitate the delay of lysosome fusion and intracellular killing of *H. pylori* ingested by macrophages. Finally, the survival rates of intracellular *capJ* knockout strain could be restored by supplementing with lysates from wild-type bacteria. Taken together, we demonstrate a positive link between rapid intracellular killing of *H. pylori* lacking cholesterol-α-glucosyltranferase by macrophage and the fusion of phagosomes with lysosomes. Our results also suggest that glucosylated cholesterols and their derivatives might play an important role in delaying the killing of macrophage which enhance bacteria evade host innate immunity.

Background and Significance

The Gram-negative bacterium *Helicobacter pylori* (*H. pylori*) colonizes the human epithelium and causes peptic ulcer, chronic gastritis, MALT-lymphoma, and adencarcinoma of the stomach [1,2,3]. Upon infection, *H. pylori* first attach to surface mucous cells of the gastric mucosa and initiate an inflammatory response in infected epithelial cells, causing peptic ulcer and gastritis[3]. However, only about 3-6% of

infected individuals have more disease progression, such as development of gastric cancer [4]. As in the case with this, *H. pylori* can be found in gland mucous cells in deeper portion of the gastric mucosa which containing high portion of mucus-associated *O*-linked glycans ([5]). Those glycans have been shown to be with potential antimicrobial activity by inhibiting the biosynthesis of a-choleseryl glucosides, a major cell wall component in *H. pylori [5,6,7]*. This biosyntheis pathway is performed by an *H. pylori* membrane-bound enzyme called CapJ (HP0421). CapJ links a glucoside with a cholesterol molecule extracted from host cell membranes and forms a-choleseryl glucosides ([6]). [7]

Cholesterol is an important structure component that modulates the fluidity of plasma membrane and is essential for the recognition of bacterial pathogens ([8,9]. In plasma membranes, cholesterol, sphingolipids, glycosylphosphatidylinositol (GPI)-anchored proteins and many signaling proteins are compartmentalized into microdomains called lipid rafts, which are resistant to solubilization in cold nonionic detergents and refer to as detergent resistant membranes (DRM)[10,11]. Lipid rafts have been known to function as platforms for localizing signaling molecules and eliciting a variety of signaling cascades [10]. Considerable evidences for the cholesterol and lipid raft dependency of attachment of bacterial pathogens suggest that the bacteria may utilize the dynamic lipid raft to facilitate the host-pathogens interactions [8]. In this regard, *H. pylori* can sense a cholesterol gradient and bind to lipid rafts in epithelial cells [12], which is required for the internalization of *H. pylori* virulence factors vacuolating toxin (VacA)[13] [14]and the translocation of a cytotoxin associated gene A (CagA) [15]. The *capJ* knockout mutant (Δ*capJ*) lacking cholesterol α-glucosyltransferase greatly lost the capability to escape phagocytosis and subsequent immune clearance responses [10,12]. One hypothesis is that cholesteryl α-glycosides may influence the membrane fluidity directly or indirectly, hence disturbing subsequent phagocytosis or downstream trafficking/signaling [16].

In this proposal, we will still focus on the host-pathogen interactions, regarding the CapJ function and the molecular mechanism of the inhibition of phagocytosis, leading to host immune evasion during *H. pylori* infection. We will purify both CapJ and its enzymatic products, cholesteryl glucosides, to study the cellular responses and signal

transduction of the host cells (macrophage cells J774A1). In addition, we will study the relationship between CapJ activity and lipid raft formation.

Specific Aims

1. Construct a knockout CapJ strain, Δ*capJ*.
2. Characterize the Δ*capJ* survival rate in macrophage J774A.1 cells
3. Investigate whether CapJ is required for absorbing cholesterol and the formation of lipid-rafts of the host-cell membrane.
4. Investigate the signaling pathway involved in the delaying of phagocytosis by macrophages in the presence of cholesteryl gulcosides.

Research Design

Since CapJ catalyzes the glycosylation of cholesterol to produce cholesteryl glucosides, which may help to evade macrophage phagocytosis and subsequent clearance in vivo [12]. Thus, the first aim is to investigate the host-pathogen interactions related with CapJ and phagocytosis by macrophage J774A.1 cells. First, using by allelic exchange mutagenesis, we disrupted the *capJ* gene in *H. pylori*, resulting a Δ*capJ* strain. In order to investigate the relationship of CapJ and intracellular survival of *H. pylori* in macrophages, we have tested the survival rate of either wild-type or Δ*capJ* ingested by macrophage J774A.1 cells (Figure 1). We found ΔcapJ is sensitive to intracellular killing by macrophage J774A.1 cells. Then, we will track the membrane trafficking molecules that may associate with the macrophage-ingested *H. pylori* [17]. Furthermore, We will investigate the molecular mechanisms of delaying phagocytosis and preventing from intracellular killing by macrophages in the presence of cholesteryl glucosides.

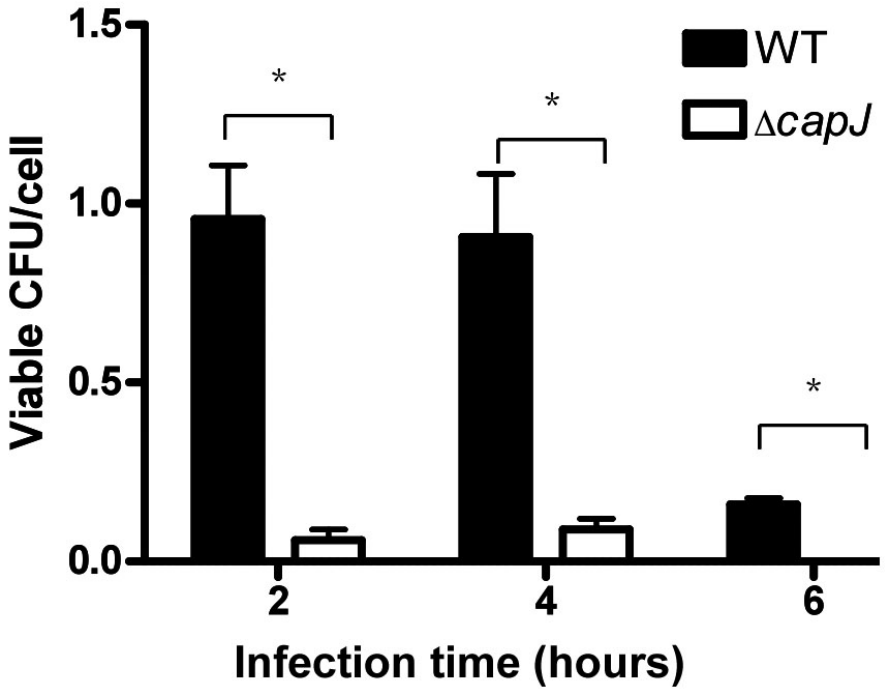

Figure 1. Intracellular survival of wild-type (WT) or capJ knockout strain (ΔcapJ) ingested by macrophage J774A.1 cells. Macrophage J774A.1 cells were infected with WT or Δ*capJ* at an MOI of 10 for indicated time periods (2, 4, and 6 hours) followed by performing gentamicin protection assay. The survival of *H. pylori* were represented as colony-forming unit (CFU) per cells.

Material and Method

H. pylori culture

Clinical isolates were recovered from frozen stocks on Wilkins-Chalgren agar with 10% sheep blood, 0.2% β-cyclodextrin, 1% IsoVitaleX, 10 mg/L nalidixic acid and 8 U/ml polymixin B in a microaerophilic atmosphere for 3–7 days at 37°C until colonies were visible. After passages onto fresh plates, the bacteria were routinely cultured on Brucella agar plates (Becton Dickinson, NY, USA) with 6 mg/L vancomycin and 2 mg/L amphotericin B. For liquid culture, the bacteria harvested from plates were cultured in Brucella broth containing 2% fetal bovine serum, 0.2% β-cyclodextrin, 1% IsoVitaleX, 6 mg/L vancomycin and 2 mg/L amphotericin B for 2 days.

Construction of Δcap J isogenic mutants

An isogenic mutant *H. pylori* is generated by insertion of the *cat* fragment into the gene of *H. pylori* through allelic replacement and selection of chloramphenicol-resistant clones[18]. A *capJ*::*cat* mutant is then generated using a natural transformation method[18].

Overexpression of CapJ

Molecular cloning experiments are performed by standard methods. For structural studies, expression and purification large amounts of target protein in a soluble, active form are required. To optimize the yield of protein expression, various conditions including vectors, hosts, and culture conditions, inducing conditions will be tested. We will first test *E. coli* expression system. Cultures of *E. coli* are grown overnight at 37ºC. Expression cultures start at OD600 = 0.05 and are grown to an OD of 0.8. Induction is performed by adding isopropyl thio-β-D-galactoside (IPTG) and further incubation for an appropriate time at 37ºC. If the protein can be expressed at a sufficiently high level and in soluble form, the clone will be used for large-scale preparation (1-5 liters). The appending tag in the expressed polypeptide makes it useful for an efficient purification of the expressed protein. The tag will then be cleaved by the designed protease and the protein will be further purified by a suitable chromatography to achieve a single homogeneous species.

In vitro CapJ enzyme assays

The CapJ activity that requires UDP-glucose can be measured by coupling assay and spectrophotometer based on the generation of UDP from UDP-glucose. Three enzymes are included: GT, pyruvate kinase, and lactate dehydrogenase. UDP that is produced by GT from UDP-glucose is used by pyruvate kinase and lactate dehydrogenase that requires NADH. The consumption of NADH can be determined by OD340 nm[17].

Gentamicin protection assay

J774A1 cells were added to mid-logarithmic phase grown bacteria at an MOI of 50 and incubated at 37°C for various periods. To determine the number of cell-associated bacteria, infected cells were washed three times to remove unbound bacteria and then lysed with distilled water for 10 min. The lysates were then diluted in PBS, plated onto Brucella blood agar plates and cultured for 4–5 days, after which the colony-forming units (CFUs) were counted.

Lipid extraction and analysis

Lipids of cells are fractionated. After pre-elution with chloroform, glycolipids are eluted with acetone:isopropanol 9:1. Lipid bands on TLC plates are visualized by dipping the TLC plates into a solution of 10% copper sulfate in 10% (v/v) H_2SO_4, drying at 100ºC and heating up 160ºC for 10 min. For NMR spectroscopy and MS, the purified glycolipids are acetylated with acetic anhydride in pyridine and subjected to preparative TLC in diethyl ether [6].

Confocal microscopic analysis of macrophage J774A.1 cells infected with H. pylori

J774A.1 cells (5×10^5 cells) were seeded on coverslips in six-well plates and incubated for 20 h. Cells were then washed and treated with or without 5.0 mMM_CD for 1 h. After three washes with PBS to remove MbCD, fresh medium supplemented with 10 _Mlovastatin was added to the cells. Cells were then not treated or infected with wild-type *H. pylori* at a multiplicity of infection (MOI) of 50 for 6 h. After three washes with PBS, cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 for 30 min, and blocked with 3% (wt/vol) bovine serum albumin in PBS for 1 h at room temperature. Samples were stained and observed with a confocal laser-scanning microscope (Zeiss LSM 510; Carl Zeiss, Go¨ttingen, Germany) with a _100 objective (oil immersion; aperture, 1.3). To observe lipid raft clustering during *H. pylori* infection, AGS cells were pre-labeled with 10ug/ml CtxB-FITC (Sigma), GM1-specific binding marker, in serum-free Hams F-12 medium at 4˚C for 30 min. Followed by washing free CtxB-FITC, AGS cells were infected with WT or Δ*capJ H. pylori* at a MOI of 10 for 3 hours. After infection, J774A.1 cells were fixed with 3.7% paraformaldehyde in 1× PBS at room temperature for 10 min, followed by permeabilization with 1x PBS supplemented with 3% donkey normal serum and 0.5% Triton X-100 for 7 min. Cells were blocked with 1X PBS supplemented with 3% donkey normal serum, 1% BSA, and 50 mM HN_4Cl for 1 hour and then incubated with anti-*H. pylori* antiserum at 4˚C overnight or at room temperature for 2 hours. Cells were then washed and incubated with Cy5-conjugated secondary antibodies (Jackson Laboratory) at room temperature for 1.5 hours. Cells were then washed, mounted, and examined with confocal laser-scanning microscopy (Zeiss LSM 510) equipped with a Plan-Apochromat 63/1.40 Oil objective. Fluorescence images were captured by using the Zeiss LSM 510 software and

processed by using LSM Image Browser (Carl Zeiss) Adobe Photoshop software.

Bacterial adherence assay

H. pylori adherence activity in J444A.1 cells was investigated using a standard assay as previously described [15]. In brief, J444A.1 cells (1.0×10^5 cells per well) grown in 6-well plates were infected with wild type or Δ*capJ H. pylori* (2.0×10^8 colony formation unit, CFU, per 0.5 ml) at a MOI of 10. The bacteria were centrifuged (500 × g for 5 min) onto the J444A.1 monolayers and then incubated at 37˚C for 3 or 6 h. To determine the number of cell-associated bacteria, infected cells were washed four times to remove unbound bacteria and then lysed with distilled water for 10 min. Lysates were diluted in PBS, plated onto brucella blood agar plates, and cultured for 4 to 5 days, after which the colony-forming units (CFUs) were counted. After washing 4 times with PBS, cells were pretreated with or without gentamycin before lysing with water. The lysates were subjected to *H. pylori* colony forming unit (CFU) assay. *H. pylori* bound on AGS cell surface was estimated by subtracting the CFU of cell pretreated with gentamycin. *H. pylori* bound on AGS cell surface is presented as CFU per cell[19].

Anticipated Results

We have obtained a *capJ* knockont mutant based on our established allelic exchange mutagenesis procedures. We also accumulated experiences on the investigating host-pathogen interactions such as the role of lipid raft formation and CagA tyrosine phosphorylation during H. pylori infection. Thus, we are confident in executing this proposal. We anticipate obtaining results as in the following:

1. We anticipate obtaining the intracellular survival rates of wild-type or Δ*capJ* ingested by macrophage J774A.1 cells
2. We anticipate understanding relationship of the CapJ avtivity and phagocytosis pathways.
3. We will perform confocal fluorescence microscopy to demonstrate the low survival rate of Δ*capJ* ingested by macrophages would be associated with the lacking of cholesteryl glucosides, which could prevent *H. pylori* from intracellular killing by macrophage cells.

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