財團法人明日醫學基金會補助專題研究計畫

☑成果報告
☐期中進度報告

探討胃幽門螺旋菌之cholesterol-α-glucosyltransferase酵素合成醣化膽固醇與逃避巨噬細胞吞噬及毒殺之關係

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

計畫類別: ☑個別型計畫　　☐整合型計畫

計畫編號:

執行期間: 民國 99 年 1 月 1 日 至 99 年 12 月 31 日

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成果報告類型 (依經費核定清單規定繳交): ☐精簡報告 ☑完整報告

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中　華　民　國　100　年　3　月　03　日

Title

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

Abstract

Helicobacter pylori (H. pylori) extracting cholesterol from the host membranes and converting into cholesteryl glucosides (CGs) that represented on its surface is associated with the function of evading host immune responses. Current studies demonstrated the innate immune responses, phagocytosis by macrophages and intracellular killing, against H. pylori infection are delayed by the activity of cholesterol-α-glucosyltranferase (HP0421, CapJ). This study, by using gentamycin protection assay, showed that CapJ-knockout mutant (ΔCapJ) is progressively engulfed and killing by phagocytic macrophages. Moreover, immunofluorescence confocal microscopy revealed that the engulfed ΔCapJ strain was significantly associated with lysosomes, supporting the promoted intracellular killing rates of the mutant in macrophages. Our results indicated that the CGs might play an important role in delaying the killing of macrophages and enhance the bacteria evade host innate immunity.

Key words: *Helicobacter pylori*, CapJ, Macrophage, Phagocytosis, lysosome

Introduction

Infection with *Helicobacter pylori (pylori)* is associated with gastric diseases including gastric ulcer, duodenal ulcer, and gastric cancer (2, 5, 6). *H. pylori* extracts cholesterol from host cell membranes and converts it into glucosyled cholesterols by a membrane-bound enzyme cholesterol-α-glucosyltranferase (HP0421, *CapJ*) (4, 8). 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 (8). However, the subsequent intracellular survival activity between the wild-type and *capJ* knockout (ΔCapJ) 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 ΔCapJ 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 ΔCapJ 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 wild type *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 CapJ activity 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.

Result and Discussion

In order to investigate the relationship of CapJ activity and intracellular survival of *H. pylori* in macrophages, we first constructed a CapJ-knockout mutant (ΔCapJ) (Figure 1) and tested the survival rate of either wild-type or Δ*capJ* ingested by macrophage J774A.1 cells by using gentamycin protection assay. The J774A1 macrophage cells were infected with wild type or ΔCapJ strains at a MOI of 10 for 2, 4, and 6 hours followed by performing colony forming assay. As shown in Figure 2, ΔCapJ is efficiently engulfed and killing by macrophage J774A.1 cells, indicating the CGs, products of CapJ, may play a protective role in evading macrophage intracellular killing. Next, we tracked the membrane trafficking molecules that may associate with the macrophage-ingested *H. pylor*. To demonstrate whether the intracellular killing is associated with phagocytic lysosome fusion with *H. pylori*, we conducted immunofluorescence microscopy to trace the engulfed *H. pylori* fused with lysosomes. Macrophage cells were pre-labeled with a lysosome-specific fluorescence dye, lysoTracker Red, at room temperature for 30 min followed by *H. pylori* infection. Following infection with wild type and ΔCapJ *H. pylori* strains, cells were fixed and stained with anti-*H. pylori* antibody. To distinguish the intra- and extra-cellular *H. pylori*, differential straining that marks *H. pylori* with the same primary antibody but with the different fluorophore-conjugated secondary antibodies at pre- and post-permeabilization with 0.1% of Triton X-100, was performed (Figure 3). Our

observation indicated that ΔCapJ but not wild type *H. pylori* were efficiently engulfed and then significantly fused with lysosomes, supporting the increased intracellular killing rates of ΔCapJ are correlated with the lysosome fusion within macrophages. These results suggested that the CapJ activity of *H. pylori* may play an essential role for evading phagocytosis and subsequently preventing intracellular killing by macrophages.

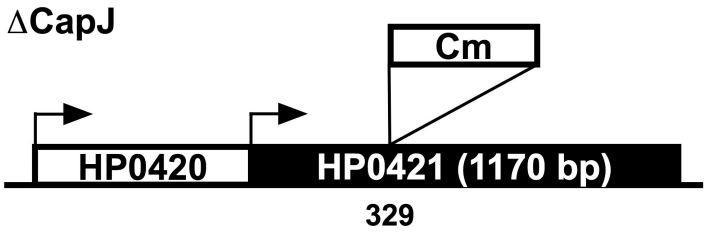

Figure 1. Schematic representation of ΔCapJ mutant construction. A cholorophenoical resistant cassette (Cm) was inserted into HP0421 (CapJ) at site 329 bp. Arrow heads indicate the gene transcription.

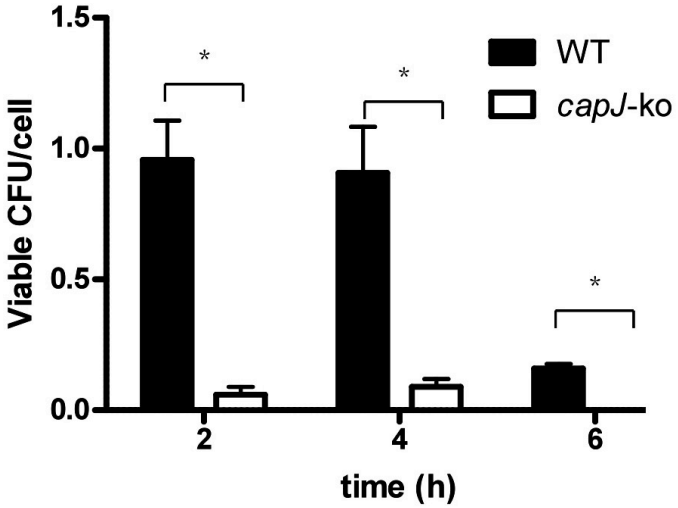

Figure 2. Intracellular survival of wild-type (WT) or capJ knockout strain (ΔcapJ) ingested by macrophage J774A.1 cells. Macrophage J774A.1 cells were infected with WT or Δ*capJ* at an MOI of 10 for indicated time periods (2, 4, and 6 hours) followed by performing gentamicin protection assay. The survival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

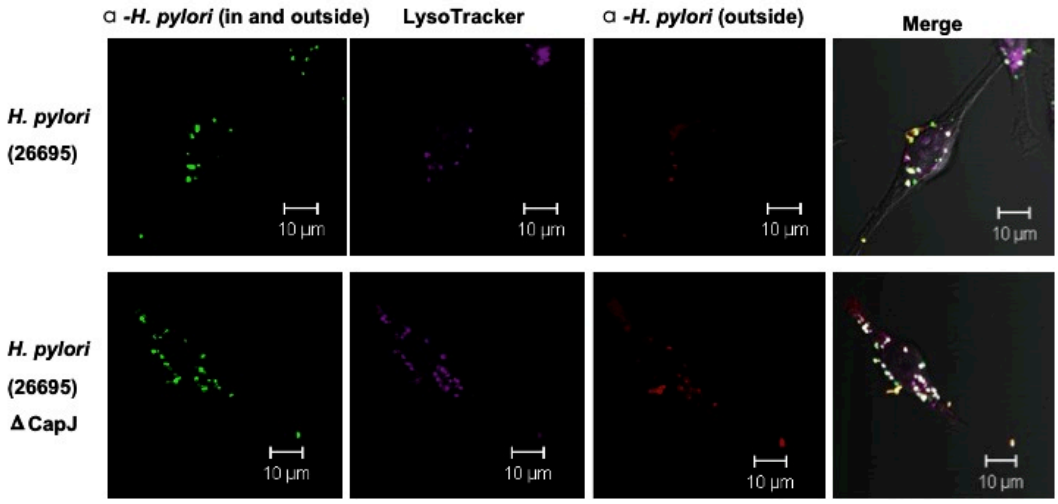

Figure 3. Confocal immunofluorescence microscopy analysis of phagocytosis. J774A1 cells were pretreated with 500 μg/ml LysoTracker Red for 1h prior to H. pylori infection. After 90 min infection with wild type (26695) or ΔCapJ *H. pylori* strains, the cells were fixed and differential stained with anti-*H. pylori* antibody to distinguish intra- and extra-cellular *H. pylori*. Before 0.1% TritonX-100 treatments, those extracellular *H. pylori* were marked with the anti-*H. pylori* antibody followed by Cy5-conjugated secondary antibody staining (Red). Subsequently, the cells were permeabilized and stained with the same anti-H. pylori antibody followed by FITC-conjugated secondary antibody staining (Green). The colocalization appears in yellow indicated the extracellular *H. pylori*. Lysotracker Red is marked in magenta. Scale bars, 10 μm.

Material and methods

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 H. pylori 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. A *capJ*::*cat* mutant is then generated using a natural transformation method described in Wang et al (7).

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.

Bacterial adherence assay

H. pylori adherence activity in J444A.1 cells was investigated using a standard assay as previously described (3). 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 (1).

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.

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