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Original paper

The CagA protein of *Helicobacter pylori* suppresses the functions of dendritic cell in mice

Hiroshi Tanaka [a], Masaru Yoshida [a,b,*], Shin Nishiumi [a,b], Naomi Ohnishi [c], Kazuki Kobayashi [a], Koji Yamamoto [a], Tsuyoshi Fujita [a], Masanori Hatakeyama [d], Takeshi Azuma [a]

[a] Division of Gastroenterology, Department of Internal Medicine, Graduate School of Medicine, Kobe University, 7-5-1 Kusunoki-cho, Chu-o-ku, Kobe, Hyogo 650-0017, Japan
[b] The Integrated Center for Mass Spectrometry, Graduate School of Medicine, Kobe University, 7-5-1 Kusunoki-cho, Chu-o-ku, Kobe, Hyogo 650-0017, Japan
[c] Division of Molecular Oncology, Institute for Genetic Medicine, Hokkaido University, Kita-15, Nishi-7, Kita-ku, Sapporo 060-0815, Japan
[d] Division of Microbiology, Graduate School of Medicine, Tokyo University, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

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ABSTRACT

CagA protein is the most assessed effecter molecule of *Helicobacter pylori*. In this report, we demonstrate how CagA protein regulates the functions of dendritic cells (DC) against *H. pylori* infection. In addition, we found that CagA protein was tyrosine-phosphorylated in DC. The responses to *cagA*-positive *H. pylori* in DC were reduced in comparison to those induced by *cagA*-negative *H. pylori*. CagA-overexpressing DC also exhibited a decline in the responses against LPS stimulation and the differentiation of $CD4^+$ T cells toward Th1 type cells compared to wild type DC. In addition, the level of phosphorylated IRF3 decreased in CagA-overexpressing DC stimulated with LPS, indicating that activated SHP-2 suppressed the enzymatic activity of TBK1 and consequently IRF3 phosphorylation. These data suggest that CagA protein negatively regulates the functions of DC via CagA phosphorylation and that *cagA*-positive *H. pylori* strains suppress host immune responses resulting in their chronic colonization of the stomach.

Introduction

Helicobacter pylori (*H. pylori*), a Gram-negative microaerophilic bacterium, chronically colonizes the gastric epithelium of more than half of all people worldwide. It is well known that *H. pylori* infection induces chronic gastritis, peptic ulcers, gastric cancer, and gastric MALT lymphoma and that the interactions among host immune responses, bacterial virulence factors and environmental factors are deeply involved in the pathogenesis of these diseases [1–4]. Although *H. pylori* infection evokes host immune responses, for example the infiltration of neutrophils, monocytes, and lymphocytes into the gastric mucosa and the induction of pro-inflammatory cytokines, *H. pylori* cannot be eliminated from the stomach in many cases [5]. However, it is unknown how *H. pylori* manages to persist in the stomach for decades.

Helicobacter pylori infection initially activates the host innate immune system. Although *H. pylori* are not able to invade into the gastric mucosa, antigen-presenting cells (APC),[1] such as dendritic cells (DC) and macrophages, recognize the antigens from *H. pylori*, and then acquired immunity is activated. Many studies have revealed that Peyer's patches (PP) play important roles in the induction of innate immunity and acquired immunity during *H. pylori* infection [6,7]. *H. pylori* is transformed to its coccoid form, which is able to access the intestinal lumen, and then is captured by DC in PP [7]. $CD4^+$ T cells activated in PP migrate to the gastric mucosa, resulting in the development of gastritis [6,7]. *H. pylori* infection predominantly induces cell-mediated immune responses. The T cells from *H. pylori*-infected gastric mucosa are Th1 cells, which secrete IFN-γ [8–10]. In addition, studies using IFN-γ −/− and IL-4 −/− mice have demonstrated the roles of Th1 cells in the development of gastritis caused by *H. pylori* infection [11]. Thus, *H. pylori* induces innate and acquired immunity.

CagA protein is one of the most studied bacterial pathogen factors of *H. pylori*. The *cagA* gene is encoded in a pathogenicity island known as '*cag* PAI' and is deeply correlated with the severity of *H. pylori*-related diseases. The injury of epithelial cells is well studied with regard to the roles of CagA in diseases evoked by *H. pylori*. After the attachment of *H. pylori* to gastric epithelial cells, CagA is translocated into gastric epithelial cells via a type IV secretion system consisting of various factors encoded in *cag* PAI [12–14]. In gastric epithelial cells, tyrosine residues on CagA are phosphorylated by src-family kinase [15,16], and the tyrosine-phosphorylated CagA forms a complex with the src homology 2 domain-containing

* Corresponding author at: Division of Gastroenterology, Department of Internal Medicine, Graduate School of Medicine, Kobe University, 7-5-1 Kusunoki-cho, Chu-o-ku, Kobe, Hyogo 650-0017, Japan. Fax: +81 78 382 6309.
E-mail address: myoshida@med.kobe-u.ac.jp (M. Yoshida).

[1] *Abbreviations used:* APC, antigen-presenting cells; DC, dendritic cells; PP, Peyer's patches; BMDC, bone marrow derived DC; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PCR, polymerase chain reaction.

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tyrosine phosphatase SHP-2, which positively regulates mitogenic signal transduction [17]. *H. pylori* is subdivided into *cagA*-positive and *cagA*-negative strains, and the induction of mucosal damage and severe atrophic gastritis by the *cagA*-positive strains are much more potent compared to those of the *cagA*-negative strains [18]. Furthermore, epidemiological studies have suggested a critical role of the *cagA*-positive *H. pylori* in the development of gastric adenocarcinoma [19,20]. In contrast to the effects of CagA in gastric epithelial cells, the roles of CagA in host immune systems remain to be elucidated. Clinically, the grade of atrophic gastritis is more severe in *cagA*-positive strains than in *cagA*-negative strains [18], and the incidence rate of gastric cancer is also higher for *cagA*-positive strains [19,20]. *cagA*-positive *H. pylori* infection elicits larger amounts of IL-8 production from epithelial cells, and then IL-8 induces cellular infiltration into the mucosa and consequently leads to the activation of anti-bacterial immune responses [21,22]. For the development of atrophic gastritis and gastric cancer, it is required that *H. pylori* colonizes the stomach for long periods and continues to damage gastric epithelial cells. CagA must be recognized as an antigen by APC and affect the host immune response because the serum from *H. pylori*-infected patients contains anti-CagA antibody [23]. However, it is unclear how CagA regulates innate and acquired immunity against *H. pylori* infection.

In this study, we investigated the influence of CagA on the function of DC. Bone marrow derived DC (BMDC) were infected with *cagA*-positive or *cagA*-negative *H. pylori* strain, and then pro-inflammatory cytokine production profiles and the degree of DC maturation were examined. *cagA*-positive *H. pylori* infection attenuated the functions of DC compared to *cagA*-negative *H. pylori* infection via tyrosine-phosphorylation of CagA. In addition, a decline in the functions of BMDC from CagA-transgenic mice was observed. Our results suggest that CagA protein suppresses host immune responses and permits *H. pylori* to colonize the stomach, resulting in continuous injury to gastric epithelial cells, which is linked to the incidence of gastric cancer.

Methods

Antibodies and reagents

LPS from *Escherichia coli* serotype 055:B5 was used (Sigma–Aldrich, St. Louis, MO). For immunoprecipitation and Western blotting, anti-CagA antibody (Austral Biologicals, San Ramon, CA), anti-IRF3 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), anti-phosphotyrosine antibody (Upstate Biotechnology, Lake Placid, NY), and anti-phospho-Ser-396 IRF3 antibody (Cell Signaling Technology, Danvers, MA) were purchased.

Bacteria

Helicobacter pylori cagA-positive strain ATCC43504 (wild type) and *cagA*-negative strain (Δ*cagA*) were cultured on blood agar plates (Nippon Becton Dickinson, Tokyo, Japan) under microaerobic conditions (5% O_2, 5% CO_2, and 90% N_2) at 37 °C. Before each experiment, *H. pylori* were grown in Brucella Broth (BD Biosciences, Franklin Lakes, NJ) supplemented with 5% equine serum for 48 h.

Preparation of DC from bone marrow

The preparation of DC from bone marrow was performed according to the method of a previous report [24]. Briefly, bone marrow cells from CagA-transgenic mice [25] or wild type C57BL/6 mice were cultured with 10 ng/ml GM-CSF and 10 ng/ml IL-4 [26]. On day 10, non-adherent cells were harvested by pipetting and then separated by density gradient centrifugation using OptiPrep (Axis-Shield, Oslo, Norway) density gradient solutions according to manufacturer's instructions. The separated cells were washed twice with PBS and then resuspended with RPMI-1640 before each experiment was performed.

Cell treatment

BMDC were infected with the *cagA*-positive or *cagA*-negative *H. pylori* at an MOI of 50:1 in serum-free medium for the periods specified in the figures and then subjected to the following experiments. AGS human gastric carcinoma cells were maintained in RPMI-1640 supplemented with 10% fetal bovine serum. After the AGS cells had been serum-starved for 12 h, the cells were infected with the *cagA*-positive or *cagA*-negative strain of *H. pylori* at an MOI of 50:1 for the periods specified in Fig. 1.

Immunoprecipitation and Western blotting

AGS cells were lysed in lysis buffer consisting of 50 mM Tris–HCl (pH 7.5), 100 mM NaCl, 5 mM EDTA, 1% Brij-35, protease inhibitors (2 mM phenylmethylsulfonyl fluoride, 10 μg/ml trypsin inhibitor, 10 μg/ml leupeptin, and 10 μg/ml aprotinin) and phosphatase inhibitors (10 mM NaF and 2 mM Na_3VO_4). For the detection of IRF3 and phosphorylated IRF3, BMDC were lysed in 100 μl of low stringency buffer consisting of 50 mM Hepes (pH 7.5), 100 mM NaCl, 10% glycerol, 1 mM EDTA, 0.5% Nonidet P-40, and the same protease inhibitors and phosphatase inhibitors. The homogenate was stirred for 1 h on ice and centrifuged at 15,000*g* for 20 min at 4 °C, and the supernatant obtained was used as a cell lysate. Immunoprecipitation using protein A/G-agarose beads (Santa Cruz Biotechnology, Inc.) was performed according to the manufacturer's protocols, and protein–antibody complexes were obtained. The cell lysates and protein–antibody complexes were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). After electrophoresis, the proteins were transferred onto a PVDF membrane (Millipore, Molsheim, France), and this was followed by the blocking of non-specific binding sites. The membrane was probed with the primary antibody before being reacted with the corresponding horseradish peroxidase-conjugated secondary antibody. The protein/antibody complex was visualized with ChemiLumiONE (Nacalai tesque, Japan) and then was detected using an Image Reader (LAS-3000 Imaging System, Fuji Photo Film).

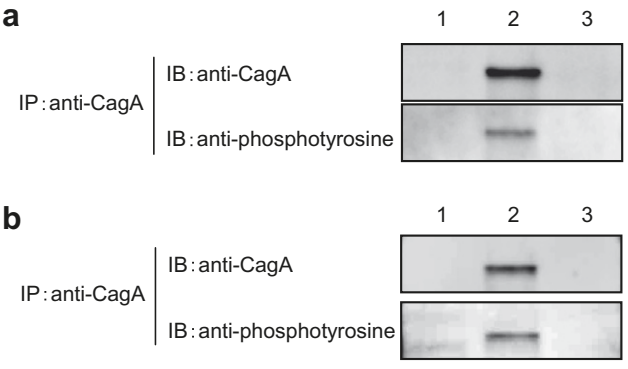

Fig. 1. The tyrosine-phosphorylation of the CagA protein in AGS cells and DC infected with *H. pylori*. AGS cells (a) and BMDC (b) were infected with the *cagA*-positive or *cagA*-negative strain of *H. pylori* at MOI of 50:1 for 5 h. Cell lysates were subjected to immunoprecipitation with anti-CagA antibody followed by Western blotting with anti-phosphotyrosine antibody. Lane 1, no infection; lane 2, infection with *cagA*-positive *H. pylori* (wild type); lane 3, infection with *cagA*-negative *H. pylori* (Δ*cagA*). Typical images are shown from at least triplicate determinations.