• *Helicobacter pylori* •

Macrophage migration inhibitory factor stimulated by *Helicobacter pylori* increases proliferation of gastric epithelial cells

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Supported by Two Competitive Earmarked Research Grant from the Research Grants Council of Hong Kong Special Administrative Region, China, No. HKU7318/01M and HKU7493/03M to Dr. HHX Xia
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Received: 2004-09-15   Accepted: 2004-11-29

Abstract

AIM: *Helicobacter pylori* (*H pylori*) is associated with increased gastric inflammatory and epithelial expression of macrophage migration inhibitory factor (MIF) and gastric epithelial cell proliferation. This study aimed at determining whether *H pylori* directly stimulates release of MIF in monocytes, whether the *cag* pathogenicity island (PAI) is involved for this function, and whether MIF stimulated by *H pylori* increases gastric epithelial cell proliferation *in vitro*.

METHODS: A cytotoxic wild-type *H pylori* strain (TN2) and its three isogenic mutants (TN2△*cag*, TN2△*cagA* and TN2△*cagE*) were co-cultured with cells of a human monocyte cell line, THP-1, for 24 h at different organism/cell ratios. MIF in the supernatants was measured by an ELISA. Cells of a human gastric cancer cell line, MKN45, were then co-cultured with the supernatants, with and without monoclonal anti-MIF antibody for 24 h. The cells were further incubated for 12 h after addition of ^{3}H-thymidine, and the levels of incorporation of ^{3}H-thymidine were measured with a liquid scintillation counter.

RESULTS: The wild-type strain and the isogenic mutants, TN2△*cagA* and TN2△*cagE*, increased MIF release at organism/cell ratios of 200/1 and 400/1, but not at the ratios of 50/1 and 100/1. However, the mutant TN2△*cag* did not increase the release of MIF at any of the four ratios. ^{3}H-thymidine readings for MKN-45 cells were significantly increased with supernatants derived from the wild-type strain and the mutants TN2△*cagA* and TN2△*cagE*, but not from the mutant TN2△*cag*. Moreover, in the presence of monoclonal anti-MIF antibody, the stimulatory effects of the wild-type strain on cell proliferation disappeared.

CONCLUSION: *H pylori* stimulates MIF release in monocytes, likely through its *cag* PAI, but not related to *cagA* or *cagE*. *H pylori*-stimulated monocyte culture supernatant increases gastric cell proliferation, which is blocked by anti-MIF antibody, suggesting that MIF plays an important role in *H pylori*-induced gastric epithelial cell proliferation.

INTRODUCTION

Migration inhibitory factor (MIF) plays a pivotal role in inflammatory and immune diseases such as septic shock, rheumatoid arthritis, atopic dermatitis, glomerulonephritis, ulcerative colitis and alcoholic hepatitis[1-4]. In our previous study, MIF was markedly up-regulated during acute gastric ulceration in rats, which was associated with accumulation of macrophages in gastric mucosa[5]. Blockade with the neutralizing anti-MIF antibody inhibited macrophage accumulation and MIF up-regulation, indicating an important pathogenic role of MIF in gastric inflammation and ulceration[5]. Recently, studies have indicated that MIF may also play a critical role as a cytokine in the development of cancers as a link between inflammation and tumorigenesis[1,6,7]. It has been reported that MIF is increasingly expressed in cells of several tumors, including melanoma, neuroblastoma, myelomonocytic leukemia, prostatic cancers of breast, colon, lung, liver, stomach and esophagus, suggesting its involvement in carcinogenesis[1,6-11], although the precise role of MIF in tumor cells remains unclear. In addition, our studies also demonstrated increased serum levels and gastric epithelial expression of MIF in gastric cancer[12].

Helicobacter pylori (*H pylori*) is a bacterium that colonizes the human stomach and causes gastric inflammation and peptic ulcer disease. Moreover, epidemiological and experimental evidence have proven that infection with *H pylori*, especially

those with *cagA* gene (a marker of the *cag* pathogenicity island (PAI)), plays an important role in gastric carcinogenesis[13-16]. We observed that *H pylori* infection increased MIF expression in both gastric inflammatory and epithelial cells[17]; thus, we hypothesize that *H pylori* infection may contribute to gastric carcinogenesis, in part, through an MIF-mediated pathway. Therefore, the aim of this study was to determine whether *H pylori* directly stimulates release of MIF in monocytes, whether the *cag* PAI is involved in this function, and whether MIF stimulated by *H pylori* increases gastric epithelial cell proliferation *in vitro*.

MATERIALS AND METHODS

H pylori strains and cell lines
H pylori strain (TN2) and its three isogenic mutants (TN2△*cag*, TN2△*cagA* and TN2△*cagE*) were used in the study. TN2, a strain positive for vacuolating cytotoxin and cag PAI, shares an ancestral strain with TN2GF4, which was previously reported to induce gastric cancer in Mongolian gerbils[16]. THP-1 (American type culture collection, ATCC, Manassas, VA, USA), a human monocyte cell line derived from acute monocyte leukemia and widely applied for in vitro experiments, was used for the determination of the effect of *H pylori* on MIF release. MKN45 (The RIKEN Cell Bank, The Institute of Physical and Chemical Research, Japan), a poorly differentiated human gastric cancer cell line with wild type p53, was used for the determination of the effect of *H pylori*-stimulated MIF on gastric cell proliferation.

Culture of bacterial cells and preparation of whole cell proteins
The bacterial cells of the strain TN2 and its isogenic mutants were cultured on blood agar plates in microaerophilic conditions, and then subcultured in liquid medium for three days, as previously described[18]. The liquid culture was transferred into a 50-mL centrifuge tube, centrifuged at 10 000 g for 10 min at 4 ℃ and washed thrice with 10 mL RPMI 1640. The pellet was suspended in 1 mL RPMI 1640, and frozen at -20℃ and thawed thrice at 37 ℃. The cells were then sonicated on ice at the maximum power for 12 cycles, with 20 s on and 10 s off in each cycle. The suspension was centrifuged at 12 000 g for 15 min at 4 ℃, and the supernatant was transferred into an Eppendorf tube, and the whole cell protein content measured using the BCA Protein Assay Kit (Gene Company Limited, Hong Kong, China). The supernatant was kept at -70 ℃ until use.

Co-culture of THP-1 with whole cell proteins of H pylori or live H pylori cells
THP-1 cells were cultured in RPMI 1640 medium (10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin and 2 μg/mL NaHCO₃) at 37 ℃ in a 50 mL/L CO₂ - 95 mL/L O₂ incubator, collected by using trypsin when they reached 80% confluence, and then adjusted to the concentration of $1×10^5$ cells/mL. The cells (100 μL) were added into wells of 24-well microtiter tray, and then incubated at 37℃ for 12 h. The whole cell protein preparations (diluted in RPMI 1640 medium with a final concentration of 40 μg/mL), live *H pylori* organisms (at organism/cell ratios of 50/1, 100/1, 200/1 and 400/1) were added to THP1 culture. Lipopolysaccharide (Sigma Chemical

Co., St. Louis, MO), known to activate THP-1 cells and stimulate MIF release[19,20], was used as a positive control, and RPMI 1640 medium was used as a negative control. In our pilot experiments, the concentration of 1 ng/mL was found to be the lowest concentration that reached the maximum stimulatory effect, and thus was used in the further study. THP1 cells were further incubated for 24 h, and culture supernatants were collected at 6, 12 and 24 h, and stored at -20 ℃ after centrifugation until use.

Measurement of MIF proteins using enzyme-linked immunosorbent assay
MIF in the supernatants was measured by an ELISA according to the manufacturer's instruction (R&D Systems, Inc., Minneapolis, MN). Briefly, 100 μL of capture anti-human MIF antibody (2 μg/mL) in phosphate-buffered saline (PBS, pH7.4) was transferred into each well of an ELISA plate, blocked with 1% BSA. Hundred microliters of each sample, or recombinant human MIF (R&D Systems, Inc.) were added, in triplicate, into corresponding wells for 2 h at room temperature. Biotinylated anti-human MIF antibody (0.2 μg/mL, R&D Systems, Inc.) was added, and incubated for 2 h at room temperature. After plates were washed thrice, 100 μL streptavidin-HRP (R&D System, Inc.) at a dilution of 1 in 200 was added into each well. Following further washing for four times, 100 μL of 1:1 mixture of reagents A (H₂O₂) and B (tetramethylbenzidine) was added. Finally, the reaction was stopped by adding 50 μL of H₂SO₄. Absorbance was read using a microtiter plate reader (NovaPath™, BIO-RAD, Hercules, CA) set at 450 nm.

Proliferation of gastric epithelial cells
After MKN-45 cells were cultured in RPMI 1640 medium at 37 ℃ for 24 h to reach a confluent monolayer, the above-prepared THP1 culture supernatants were added with a final concentration of 20% in the medium, with or without monoclonal anti-MIF antibody (1 ng/mL, R&D System, Inc.). RPMI 1640 medium was used as a negative control. The cells were further incubated for 24 h, and then ³H-thymidine (50 μc) was added to the medium, and the cells were incubated for additional 12 h. The levels of incorporation of ³H-thymidine of the MKN-45 during the last 12 h were determined in a liquid scintillation counter (Beckman LS 6 500, Beckman Instruments, Inc., Fullerton, CA) as counts per minute (cpm).

Statistical analysis
Data obtained from the study were expressed as the mean±SD. Statistical analysis was performed using SPSS software (version 10.0, SPSS Inc., Chicago, IL). Independent samples *t*-test, or the Mann-Whitney *U* test, where appropriate, was used to determine the differences in numeric variables. *P* values calculated were two-tailed. The alpha level of significance was set at *P*<0.05.

RESULTS

Effect of H pylori on the MIF expression
Secretion of MIF by THP-1 cells was significantly increased by the whole cell proteins of the wild-type TN2 strain, *cagA*-
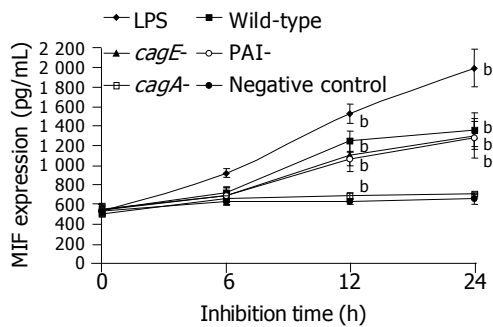
Figure 1 Expression of MIF by THP1 after co-culture with LPS (1 ng/mL) and whole cell proteins (40 μg/mL) of wild-type cytotoxic *H pylori* strain TN2 and its isogenic mutants, TN2△cag (PAI-), TN2△cagA (cagA-) and TN2△cagE (cagE-). The bars represent the standard deviations. [b]P<0.01, compared with the control.

Figure 2 Expression of MIF by THP1 after co-culture with a wild-type cytotoxic *H pylori* strain TN2 and its isogenic mutants, TN2△cag (PAI-), TN2△cagA (cagA-) and TN2△cagE (cagE-), at different organism/cell ratios for 12 h. The bars represent the standard deviations. [b]P<0.01, compared with the isogenic mutant TN2△cag (PAI-).

deleted or *cagE*-deleted mutants in a time-dependent manner (Figure 1). However, the whole cell proteins of PAI-deleted mutants did not have the stimulatory effect on MIF secretion (Figure 1). Similarly, co-culture of live organisms of the wild-type strain, *cagA*-deleted or *cagE*-deleted mutants significantly increased MIF secretion in a dose-dependent manner (Figure 2). However, the PAI-deleted mutant had no effect on MIF secretion (Figure 2).

Effect of H pylori-stimulated MIF on gastric epithelial cell proliferation

Proliferation of MKN-45 cells was significantly increased by adding the supernatant of co-culture of THP-1 with LPS or with the wild-type strain (Figure 3). The supernatant of co-culture of THP-1 with the PAI-deleted mutant only slightly increased cell proliferation (Figure 3). Interestingly, addition of monoclonal anti-MIF antibody decreased cell proliferation of the gastric cancer cells. Furthermore, the stimulatory effect of LPS and the wild-type *H pylori* strain on the cell proliferation was completely abolished by the addition of the antibody (Figure 3).

DISCUSSION

The present study shows that either the whole cell proteins or live organisms of the cytotoxic *H pylori* strain stimulate the monocytes to release MIF. In addition, the *cag* PAI-deleted mutant has little effect on the MIF release, suggesting the essential role of PAI in the stimulation of MIF release. Moreover, the supernatant of *H pylori* co-cultured monocytes increases proliferation of gastric cancer cells and neutralizing monoclonal anti-MIF antibody reduces cell proliferation, indicating that MIF plays an important role in gastric cell proliferation.

Studies have shown that MIF is a pro-inflammatory cytokine and is constitutively expressed, stored in a preformed manner in cytoplasm, and released into circulation upon stimulation[21]. It has been reported that normal gastric epithelial cells constitutively, albeit weakly, express MIF[5,17,22]. In our previous study, we observed that MIF expression was significantly increased in gastric epithelial cells in the presence of *H pylori* infection[17]. More importantly, it is well known that
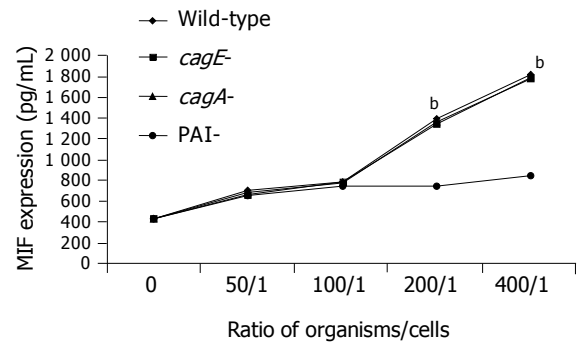
preformed MIF protein is present in both resting macrophages and monocytes, indicating that the macrophage is not only a target, but also a source of MIF *in vivo*[2,21,23,24]. It is well established that *H pylori* infection is associated with gastric infiltration of acute and chronic inflammatory cells, including microphages and monocytes. Previously, we applied a double staining technique to simultaneously detect activated T cells (CD45RO) and macrophages (KP-1) and observed increased MIF expression in these cells in gastric mucosa[17]. In present *in vitro* study, we clearly show that *H pylori* stimulated release of MIF by monocytes, most likely through its *cag* PAI. However, both *cagA* and *cagE* appeared not to play a role in the stimulatory effect, since *cagA*- or *cagE*-deleted mutant possess the similar stimulatory effect on MIF release to their parent. MIF may play an important role in gastric inflammation and injury. We hypothesize that colonization of *H pylori* in the stomach directly stimulates the gastric epithelial cells to release MIF, which then promotes local infiltration of inflammatory cells including neutrophils, T- and B-lymphocytes and macrophages, resulting in the production of inducible nitrite oxide synthase (iNOS) and cytotoxic cytokines such as TNF-α, IFN-γ and ICAM-1[1,5,19,25,26]. Consequently, activated T-cells and macrophages per se release a large amount of MIF. In the presence of chronic *H pylori* infection, this cycle continues, which contributes to more severe inflammation, and gastric injury such as gastric erosion and ulcerations.
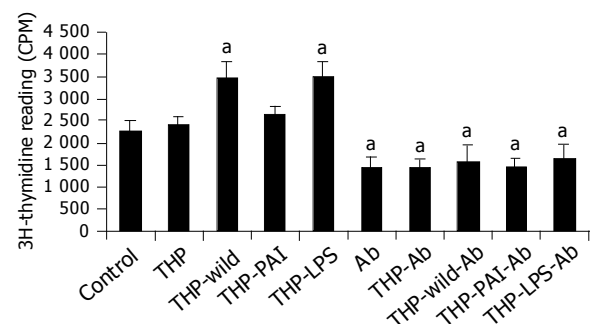

Figure 3 Effect of LPS and *H pylori*-co-cultured supernatants of THP1 cells, with and without addition of monoclonal anti-MIF antibody (Ab), on the cell proliferation of MKN-45. The bars represent the standard deviations. [a]P<0.05 *vs* the control.

Recently, we demonstrated that MIF expression was significantly increased in precancerous lesions and gastric cancer, suggesting its role in gastric carcinogenesis[12]. MIF could be involved in carcinogenesis, by promoting cell proliferation, tumor angiogenesis and metastasis [1,8,26-30]. In previous animal studies, it was observed that increased MIF expression was associated with enhanced proliferation of murine colon cancer cells in response to growth factors and neutralizing anti-MIF-antibodies dramatically reduce the initial outgrowth of 38C13 B cell lymphoma cells in mice[26,27]. MIF has been identified as an angiogenic factor and neutralization of MIF by anti-MIF antibodies inhibits endothelial cell growth and leads to a reduced number of tumor capillaries[27,28]. Inhibition of angiogenesis by anti-MIF antibody treatment has also been reported in a human melanoma model and in murine colon carcinoma cells[8,26,29]. Both increased proliferation of tumor cells and angiogenesis would inevitably result in metastasis. In the present study, we observed that supernatant of monocytes after co-culture with *H pylori* increased proliferation of gastric cancer cells, and addition of anti-MIF antibody blocked the increase in cell proliferation, suggesting MIF stimulated by *H pylori* plays an important role in gastric epithelial cell proliferation. How MIF regulates cell proliferation is under intensive investigation. Recent studies suggest that modulation of cell proliferation by MIF could involve a complex regulatory system, in which proteins p53, JAB/CSN5/p27^{Kip1} and ERK1/2, and possibly other signalosome proteins, may play important roles[1,6,7,30,31].

Genetic analysis reveals that *H pylori* strains can be divided into two types, by the presence or absence of a 40 kb of foreign DNA, i.e., the *cag* PAI which consists of at least 18 open-reading frames such as *cagA*, *cagE*, *etc.*[32]. Over the past decade, *cagA*, a 120-128 ku protein, has been identified as a marker for the presence of *cag* PAI[33,34]. Upon contact with gastric epithelial cells, *cagA* is translocated into gastric epithelial cells, where *cagA* undergoes tyrosine phosphorylation and recruits and activates Src homology phosphatase 2, thereby inducing a growth-factor-like effect[34]. Recent studies have indicated that *cagE* and several other genes of *cag* PAI code a type IV secretion system, which is involved in the translocation of *cagA* protein into the gastric epithelial cells[34-36]. Moreover, isogenic mutant studies demonstrated that some *cag* PAI genes, including *cagE*, are essential for interleukin-8 induction and nuclear factor-κB activation[32,34,37,38]. However, a recent study found that the association between *cagE* genotype and IL-8 induction was weak and implied that gastric cell factors or other bacterial factors are involved in the induction of IL-8[39]. In animal studies, it was observed that knocking out of *cagE* gene deprived wild-type *H pylori* of the pathogenicity for gastritis and gastric ulcer in Mongolian gerbils, suggesting that this gene is responsible for various gastric lesions[40,41]. Day *et al*[42], reported that *cagE* is associated with *H pylori*-induced duodenal ulceration in children in Canada. However, studies in Japan and Taiwan showed that presence of *cagE* appeared not to be related to the severity of gastric mucosal inflammation and ulceration in patients with upper gastroduodenal symptoms since almost all isolates were *cagE* positive[43-45]. In the present study, we demonstrated that *cag* PAI is essential for the stimulation of MIF secretion.

However, neither *cagA* nor *cagE* is involved in the stimulation, suggesting that genes other than *cagA* and *cagE* in the *cag* PAI are responsible for this function.

In conclusion, *H pylori* stimulates MIF release in monocytes, likely through its *cag* PAI, but not related to *cagA* and *cagE*. *H pylori*-stimulated monocyte culture supernatant increases gastric cell proliferation, which is blocked by anti-MIF antibody, suggesting that MIF plays an important role in *H pylori*-induced gastric epithelial cell proliferation.

ACKNOWLEDGMENTS

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