Extracellular galectin-3 counteracts adhesion and exhibits chemoattraction in Helicobacter pylori-infected gastric cancer cells

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Galectin-3 (Gal-3) is a β-galactoside lectin that is upregulated and rapidly secreted by gastric epithelial cells in response to Helicobacter pylori infection. An earlier study reported the involvement of H. pylori cytotoxin-associated gene A (cagA) in the expression of intracellular Gal-3. However, the role of extracellular Gal-3 and its functional significance in H. pylori-infected cells remains uncharacterized. Data presented here demonstrate secretion of Gal-3 is an initial host response event in gastric epithelial cells during H. pylori infection and is independent of CagA. Previously, Gal-3 was shown to bind to H. pylori LPS. The present study elaborates the significance of this binding, as extracellular recombinant Gal-3 (rGal-3) was shown to inhibit the adhesion of H. pylori to the gastric epithelial cells. Interestingly, a decrease in H. pylori adhesion to host cells also resulted in a decrease in apoptosis. Furthermore, the study also demonstrated a chemoattractant role of extracellular rGal-3 in the recruitment of THP-1 monocytes. This study outlines the previously unidentified roles of extracellular Gal-3 where it acts as a negative regulator of H. pylori adhesion and apoptosis in gastric epithelial cells, and as a chemoattractant to THP-1 monocytes. Our findings could contribute to the better understanding of how Gal-3 acts as a modulator under H. pylori-induced pathological conditions.

INTRODUCTION

Helicobacter pylori is a major risk factor for the development of peptic ulcer disease and gastric cancer (Blaser, 1998; Uemura et al., 2001). Virulent H. pylori strains carry the cag pathogenicity island, which encodes the type IV secretion system (TFSS) (Odenbreit et al., 2000). The bacterium colonizes the human gastric mucosa and adheres to the underlying epithelium, thereby establishing a stable infection (Subhash & Ho, 2015). Adhesion of H. pylori to the gastric epithelium facilitates translocation of CagA oncoprotein into the host cells through the TFSS. Once translocated into the host cells, CagA induces a humming bird phenotypic change in cultured AGS gastric cancer epithelial cells and also triggers a series of intracellular signalling pathways (Higashi et al., 2002; Mimuro et al., 2002). Another virulence factor that has been implicated to play a contributory role in H. pylori pathogenesis is LPS. Although the LPS of H. pylori exhibits low endotoxic and immunological activity (Moran et al., 2002), its O-antigen side chain shows structural similarity to Lewis X antigen. This facilitates the interaction between H. pylori LPS and specific receptors on the host cell surface, and thereby enhances the adhesion of the bacteria to the gastric epithelium (Mahdavi et al., 2003; Owen et al., 2001). Barondes et al., (1994) showed that H. pylori O-antigen side chain functions as a ligand for galectin 3 (Gal-3).

Gal-3 is a 31 kDa β-galactoside lectin highly expressed in human gastric epithelial cells that exists in both intracellular and extracellular forms. The localization and subcellular expression was shown to be dependent on the tissue, cell type, proliferative state and level of differentiation (Okada et al., 2006). An increase in Gal-3 expression is linked to increased malignancy in gastric adenocarcinomas and in a number of tumours (Baldus et al., 2000; Miyazaki et al., 2002). Interestingly, Gal-3 is upregulated and rapidly secreted as a host response to H. pylori infection (Fowler et al., 2006). Previously, Gal-3 was reported as a negative regulator of LPS function in Salmonella infection. Moreover, blocking of Gal-3 with neutralizing antibodies or its high-

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Abbreviations: Gal-3, galectin 3; CFDA-SE, carboxyfluorescein diacetate succinimidyl ester.

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affinity ligand β-lactose led to an increase in LPS sensitivity and inflammatory cytokine production by macrophages (Li et al., 2008). Although Gal-3 reportedly has various roles in cancers and infections, no prior work has been done to identify its functional significance in H. pylori-induced infections. Given its diverse functions, it is therefore not surprising that Gal-3 is implied to potentially play a role in contributing to H. pylori-induced gastric malignancies. The study presented here investigates the role of extracellular Gal-3 in host response to H. pylori infection. Our results identify extracellular Gal-3 as a negative regulator of H. pylori adhesion and apoptosis in gastric epithelial cells. The current study also suggests that extracellular Gal-3 may act as a chemotraactant in triggering the recruitment of THP-1 monocytes during H. pylori infection.

METHODS

H. pylori and AGS cell culture. H. pylori 26695 WT and its cagA iso-
genics strain (ΔcagA) expressing a chloramphenicol resistance gene cassette. H. pylori ΔcagA was constructed using a modified multi-
step polymerase chain reaction (PCR) approach (Gong et al., 2010). Bacteria were cultured on chocolate blood agar (Oxoid) supplemented with 5 % horse blood (Thermo Scientific). Plates were incubated at 37 °C under micro-aerobic conditions (10 % CO₂, 7.5 % O₂, 82.5 % N₂).

AGS gastric cancer epithelial cells (CRL-1739; American Type Culture Collection) and THP-1 human acute monocytic leukaemia cells (TIB-202; American Type Culture Collection) were cultured in Ham’s F12K (Sigma-Aldrich) and DMEM medium (Hyclone), respectively, supplemented with 10 % FCS (Gibco). The cultures were incubated at 37 °C in a CO₂ water-jacketed incubator (Thermo Scientific) with 95 % humidity and 5 % CO₂.

Detection of extracellular Gal-3 expression. The assay for Gal-3 release was adapted from the method of Zhu & Ochieng. (2001). AGS cells (1 × 10⁶ cells ml⁻¹) were seeded into F12K medium in a 75 cm² tissue culture flask (Sigma-Aldrich). Upon reaching 80 % confluency, cells were serum starved for 4 h before being infected with H. pylori at an m.o.i. of 100:1. The culture supernatant was collected at specific time intervals. Detection of Gal-3 expression in the H. pylori-infected AGS cell culture supernatants was carried out using Western blot. Briefly, the culture supernatants were subjected to PAGE and protein bands were electro-transferred onto PVDF membrane (BioRad). The membrane was blocked with 2 % PBS–BSA (Merck) overnight at 4 °C. To detect Gal-3 expression, blocked membranes were incubated with rat-anti-Gal-3 mAb (1:50 dilution; Santacruz) in 2 % PBS–BSA for 2 h at 37 °C. Following three washes in PBS containing 0.1 % (v/v) Tween 20 (PBST), the blots were incubated with goat anti-rat immunoglobulin G conjugated to alkaline phosphatase (1:1000; DakoCytomation) in 2 % PBS–BSA for 2 h at 37 °C. For detection of the proteins, an ECL detection assay (Thermo Scientific) was performed according to the manufacturer’s instructions.

Adhesion assay. Detection of H. pylori adhesion to AGS cells was performed as previously described (Guzman-Murillo et al., 2001). H. pylori was biofilm-labelled by dissociating N-biotin-N-hydroxysuccinimide ester (Biotin-NHS, Roche) in DMSO (1 µg ml⁻¹). The solution was then mixed with H. pylori suspension (1 × 10⁶ ml⁻¹) at a ratio of 1:100. AGS cells (1 × 10⁴ ml⁻¹) were seeded in a 24-well plate and incubated until 80 % confluency in serum-free medium. The cells were then further cultured with varying concentrations of human recombinant Gal-3 (rGal-3; ebioscences) ranging from 0 to 10 µg ml⁻¹, followed by 2 h of incubation in a suspension of biotin-labelled H. pylori cells at 37 °C. An aliquot of 250 µl HRP-conjugated streptavidin (Roche) was then added to each well and the plates were incubated for a further 90 min at 37 °C. After washing the plates three times with PBS, 50 µl O-phenylenediamine-dichloride (Sigma) was added to each well and the plates were incubated for another 20 min in the dark. The reaction was stopped with the addition of 100 µl 2 M sulphuric acid (Merck) and colour development was measured at 495 nm using a Tecan Infinite plate reader. The adhesion of biotin-labelled H. pylori to AGS cells was expressed in terms of OD units.

Apoptosis assay. Apoptosis was detected by an Annexin V-FITC kit (BD Pharmingen) according to the manufacturer’s instructions. Briefly, AGS cells (1 × 10⁶ cells ml⁻¹) were grown to 80 % confluency in 25 cm² flasks in F12K medium supplemented with 10 % FCS. Prior to infection, the medium was replaced with serum-free F12K. Then the AGS cells were co-cultured with H. pylori at 37 °C. After 24 h of incubation, cells were harvested and washed three times with cold PBS before being resuspended in 1 × binding buffer. An aliquot of 100 µl cell suspension was transferred into a microfuge tube, and mixed with equal volumes (5 µl) of annexin V-FITC and propidium iodide (PI). The cells were then analysed using a flow cytometer (Cyan ADP; Beckman Coulter) within 1 h. The data obtained were analysed using Summit 4.3 (Beckman Coulter).

Migration assay. THP-1 monocytes were labelled by carboxyfluorescein diacetate succinimidyl ester staining (CSDA-SE) according to the product recommendation (Molecular Probes). A 5 µM working concentration of carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) was added to 1 ml of the THP-1 cell suspension (10⁶ cells ml⁻¹) in DMEM (HyClone) medium. The suspension was mixed thoroughly and labelled by incubating the mixture at 37 °C for 10 min. An equal volume of DMEM medium was then added to quench the labelling process. The cells were then collected by centrifugation at 1000 g for 10 min at 4 °C. The cell pellet was washed twice in 2 ml DMEM and resuspended in DMEM for subsequent experiments. AGS cells (1 × 10⁵) were seeded in the lower chamber of a Costar Transwell migration plate with pore size of 4 µm. Upon reaching 80 % confluency, CFDA-SE-labelled THP-1 monocytes were added onto the Transwell inserts. After 12 h of incubation, the number of THP-1 cells that migrated across the Transwell chamber towards the AGS cells were enumerated by random selection of 10 fields under a fluorescence microscope (Olympus). Furthermore, the Transwell microporous membrane was then excised and viewed under CLSM with Z-stacking of the filter planes. Images were then analysed using Imaris version 7.3 (Bitplane).

RESULTS

Detection of extracellular Gal-3 expression

Studies have shown that the capacity to secrete extracellular Gal-3 varies from cell line to cell line, and is also dependent on various external stimuli, including adhesion, membrane fusion events, heat shock and calcium ionophores (Hughes, 1999; Sato & Hughes, 1994). The current study sought to determine the effect of extracellular Gal-3 in H. pylori-infected AGS cells. Caga deletion in H. pylori 26695 was confirmed by PCR and Western blotting (Fig. S1, available in the online Supplementary Material). The primers used for caga amplification are described in Table S1. The presence of O-antigen side chain in H. pylori 26695 was also determined (Table S2, Fig. S2). Consistent with the previous
study (Fowler et al., 2006), secretion of Gal-3 in *H. pylori*-infected AGS cells was observed as early as 30 min. Fig. 1 shows the level of expression of extracellular Gal-3 in AGS cells infected with WT and ∆cagA strains of *H. pylori*. Gal-3 expression remained stable throughout 24 h post-infection and was found to be independent of *H. pylori* CagA. A basal level of extracellular secreted Gal-3 was observed in the uninfected cells.

**Role of extracellular Gal-3 in *H. pylori* adhesion**

The effect of extracellular Gal-3 on *H. pylori* adhesion was investigated using a biotin–streptavidin binding assay as described elsewhere (Guzman-Murillo et al., 2001). Through dose-dependent analysis, a concentration of 8 µg ml⁻¹ rGal-3 was found to be optimal in causing maximum decrease of *H. pylori* adhesion to AGS cells (Fig. 2a). *H. pylori* WT adhered to the AGS cells within 2 h of incubation. Intriguingly, a 22.1% decrease in overall adherence was observed in the presence of rGal-3 (P<0.005) as compared with the mock (PBS)-treated cells (Fig. 2b). On the other hand, neutralization of extracellular Gal-3 using anti-Gal-3 mAb resulted in 10.1% increase in the adhesion of *H. pylori* to AGS cells (P<0.005).

**Effect of extracellular Gal-3 in *H. pylori*-induced apoptosis**

The regulatory effect of Gal-3 in *H. pylori*-induced apoptosis was determined in this study. As illustrated in Fig. 3, the percentage of annexin V-labelled apoptotic cells was 23.8% after infection with *H. pylori* WT for 24 h. Interestingly, *H. pylori*-infected AGS cells co-incubated with rGal-3 showed a reduction in apoptosis (12.35%) as compared with the untreated cells, hence suggesting Gal-3 as a negative regulator of apoptosis in *H. pylori*-infected cells. Conversely, co-incubation of *H. pylori*-infected cells with anti-Gal-3 mAb resulted in partial neutralization of the inhibitory effects of Gal-3, thereby causing an increased sensitivity to apoptosis (16.42%).

**Role of Gal-3 as chemoattractant of THP-1 monocytes**

Using a Transwell migration chamber, rGal-3 was shown to induce migration of THP-1 monocytes in a dose-dependent manner (Fig. 4a). Presence of rGal-3 significantly increased THP-1 migration (P<0.05) at concentrations >0.5 µM compared with diluent control and showed a bell-shaped dose-dependent pattern (control, 13.67±2.08; 0.1 µM, 29±3.60; 0.5 µM, 39±4.19; 1 µM, 49±5.07; 2 µM, 69±6.12).

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**Fig. 1.** Secretion of extracellular Gal-3. AGS cells were infected with *H. pylori* WT and ∆cagA strains. The culture supernatants were collected at specific time intervals and the presence of extracellular Gal-3 was detected by Western blot using anti-Gal-3 mAb.

**Fig. 2.** Role of extracellular Gal-3 in *H. pylori* adhesion. (a) Dose-dependent effect of rGal-3 in *H. pylori* adhesion. AGS cells treated with varying concentrations of rGal-3 were infected with biotin-labelled *H. pylori* WT. (b) Effect of rGal-3 on *H. pylori* adhesion. AGS cells were pre-treated with either r-Gal-3 (functioned as extracellular Gal-3) or anti-Gal-3 mAb for 2 h. These cells were then infected with biotin-labelled *H. pylori* WT. The amount of bacterial adhesion was quantified by a biotin–streptavidin-based bacterial adhesion assay. *H. pylori*-infected cells without r-Gal-3 served as control. The data are representative of three separate experiments and the error bars represent SD of triplicate samples. *P<0.05 and **P<0.005 were considered significant.
The interactions between various host and microbial proteins result in chronic infection, where enhanced inflammation and proliferation have been reported as major risk factors (Moss & Blaser, 2005). Gal-3 is a widely studied multi-functional lectin, and changes in its expression levels have been reported in microbial infections and cancers (Yang et al., 2008) The implication of Gal-3 during malignancy progression has been suggested in the development of various types of cancers, including gastric cancer (Miyazaki et al., 2002).

Atypically, Gal-3 lacks the classical secretion signal sequence and does not pass through the standard ER/Golgi pathway. However, studies have shown that secretion of extracellular Gal-3 occurs through a non-classical pathway (Hughes, 1999; Menon et al., 2011). Importantly, Iurisci et al. (2000) have reported significantly high levels of Gal-3 in the sera of patients with breast, lung, ovarian and gastrointestinal cancers. Consistent with the findings of Fowler et al. (2006), the present study demonstrated a rapid secretion of extracellular Gal-3 in H. pylori-infected cells. Notably, the amount of secreted extracellular Gal-3 remained consistent over a period of up to 24 h post-infection and was found to be independent of CagA. The data suggest that secretion of extracellular Gal-3 is an initial event in H. pylori-infected cells and is likely to be attributable to an immediate host response mechanism against the pathogen. This assumption is well supported by previous studies, which suggest

DISCUSSION

The consequences of H. pylori infection range widely from asymptomatic colonisation to severe gastric malignancies. The interactions between various host and microbial proteins result in chronic infection, where enhanced

Fig. 3. Flow-cytometric analysis of the role of extracellular Gal-3 in H. pylori-induced apoptosis. AGS cells were co-incubated with either anti-Gal-3 mAb or rGal-3 and then infected with H. pylori WT. (a) Uninfected cells (top row) served as experimental control. The percentages of apoptotic cells (lower right quadrant) were then determined by annexin V-FITC-based apoptosis assay and compared against different treatment conditions. (b) Mean percentage of apoptotic cells from three separate experiments. Error bars represent SD of triplicate samples. *P<0.05 was considered significant. R8, R9, R10 and R11 represents the quadrants of PI and FITC fluorescence. FITC, fluorescein isothiocyanate; log comp, logarithmic scale with compensation applied and PE, phycoerythrin.

0.5 µM, 63.7±3.05; 1 µM, 93.33±6.65; 2.5 µM, 135.30±7.50; 5 µM, 177.33±3.21; 7 µM, 138.33±4.93; 10 µM, 126.6±5.68; n=4 experiments). The role of H. pylori in inducing secretion of Gal-3 and migration of THP-1 cells across the Transwell chamber was confirmed by CFDA-SE staining of THP-1 cells. The Transwell membrane was excised and the chamber harbouring AGS cells. No migration was observed in the absence of either rGal-3 or anti-Gal-3 mAb. Similarly, infection of AGS cells with H. pylori WT also resulted in active migration of THP-1 cells across the filter membrane.

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secreted Gal-3 as a pro-inflammatory signal that activates NADPH oxidase (Karlsson \textit{et al.}, 1998) and superoxide production from neutrophils (Yamaoka \textit{et al.}, 1995). As an extracellular protein, Gal-3 can interact with the glycoproteins within the extracellular matrix or can act as a ligand to cross-link carbohydrates of surface proteins by N-terminal oligomerization (Li \textit{et al.}, 2008). In addition, Gal-3 was also shown to have a binding affinity towards β-galactoside carbohydrates, which are common structures on the LPS of many pathogens (Mandrell \textit{et al.}, 1994). Of interest, \textit{H. pylori} LPS is characterized by a long O-antigen side chain that comprises a polymer of N-acetyl lactosamine, a known ligand for Gal-3 (Sato & Hughes, 1992). H. pylori 26695 was shown to express O-antigen side chain in laboratory culture conditions and binding of Gal-3 to the O-antigen side chain of \textit{H. pylori} LPS has been previously reported (Monteiro \textit{et al.}, 2000; Fowler \textit{et al.}, 2006). However, the significance of this binding in the pathogenicity of \textit{H. pylori} infection is not fully understood. The ability of \textit{H. pylori} to adhere and colonize the gastric epithelium is believed to play a central role in gastric infections (Su \textit{et al.}, 1999). Since Gal-3 exhibits N-terminal and C-terminal binding, a cross-linking property was previously attributed to its extracellular expression (Nangia-Makker \textit{et al.}, 2008). On account of this, the current study provides a deeper understanding of the extracellular roles of Gal-3. Interestingly, the presence of rGal-3 in the cell culture medium induced a dose-dependent reduction in \textit{H. pylori} adhesion, suggesting its inhibitory role. Adding further credence to its inhibitory role, neutralization of extracellular Gal-3 using anti-Gal-3 mAb resulted in an increase in \textit{H. pylori} adhesion to AGS cells. The inhibitory effect of extracellular Gal-3 appears in contrast with the function of membrane-bound Gal-3 shown previously in the adhesion of \textit{Klebsiella pneumoniae} (Mey \textit{et al.}, 1996), \textit{Pseudomonas aeruginosa} (Gupta \textit{et al.}, 1997) and \textit{Neisseria gonorrhoeae} (John \textit{et al.}, 2002) to their target cells. However, the reverse trend observed in the present study could be related to the differences in LPS composition of \textit{H. pylori}, the localization of Gal-3 (whether cell-surface-bound or extracellular) and also the variations in target cell lines. The concentrations of rGal-3 used in our assays were higher than the amounts secreted during infection under culture conditions (Fig. S3). However, the rGal-3 concentrations tested in the current study are considered physiologically relevant, as a previous study has identified Gal-3 concentrations up to

Fig. 4. Gal-3-induced migration of THP-1 cells. (a) Various concentrations of rGal-3 (0–10 µM) were applied to the lower chambers of a Transwell migration apparatus while THP-1 monocytes were applied to the upper chambers. The migration assay was performed to enumerate the number of cells that migrated from the upper chamber to the lower chamber, using phase-contrast microscopy. The number of THP-1 cells that migrated across the Transwell chamber towards AGS cells was counted using a fluorescence microscope and data are plotted as the mean of the number of cells in three randomly selected fields. Data are presented as mean±SD. *P<0.05 was considered significant. (b) AGS cells were seeded into the lower wells of a Transwell migration chamber and were incubated with either rGal-3 or \textit{H. pylori} WT. Untreated AGS cells served as experimental control. THP-1 cells were stained with CFDA-SE fluorescent dye (green). The cells migrating across the filter membrane were detected by excision and fixation of the filter, followed by confocal microscopic analysis.
950 ng in the sera of gastric cancer patients as compared with a maximum lower concentration of 180 ng in healthy individuals (Iurisci et al., 2000). Apart from adhesion, extracellular Gal-3 was shown to be a negative regulator of H. pylori-induced apoptosis. The data do not implicate Gal-3 as an anti-apoptotic molecule by itself, but its binding to H. pylori LPS and subsequent interference with adhesion of the bacteria to the receptor sites on gastric epithelial cells may inadvertently result in a down-regulation of apoptosis. Although anti-apoptotic functions of intracellular Gal-3 have been reported in many cell lines and cancers, little evidence is available regarding its extracellular roles. By identifying the regulatory role of extracellular Gal-3 in apoptosis, the current study provides further understanding of the role of Gal-3 in host response to H. pylori infection.

Using in vitro migration assays, extracellular rGal-3 has been shown to promote monocyte and macrophage migration (Sano et al., 2000). Studies by Bhaumik et al. (2013) reported neutrophil recruitment by Gal-3 during early stages of Leishmania infection. It was suggested that the rapid release of Gal-3 from infected cells may represent an early warning system and instigate the immediate trafficking of phagocytic cells to the site of infection (Fowler et al., 2006). Since mononuclear migration is a characteristic event in H. pylori-induced inflammation (Mori et al., 2001), the current study investigated a chemoattractant role of secreted Gal-3 on monocytes. The study employed a Transwell migration chamber assay that utilized CFDA-SE staining of THP-1 monocytes for detection using confocal microscopy. The co-incubation of AGS cells with H. pylori could induce the migration of THP-1 monocytes towards the site of infection. Of note, THP-1 cell migration was not found to be inhibited by addition of Gal-3 mAb to H. pylori-infected AGS cells. (Fig. S4), hence implicating other molecules that could also contribute to the chemotraction of THP-1 cells. In contrast, absence of H. pylori induced no THP-1 cell migration. Interestingly, the addition of extracellular recombinant Gal-3 could rescue THP-1 migration towards uninfected AGS cells in a dose-dependent manner. Thus, the data not only imply Gal-3 induced monocyte migration but also suggest its role as a major chemoattractant, since a significantly high number of THP-1 cells migrated in the presence of recombinant Gal-3. It is therefore proposed that extracellular Gal-3 could play a role in host innate immune response, where its secretion would serve as an indicator of chronic inflammation during H. pylori infections. Of note, THP-1 cell migration was not found to be inhibited by addition of Gal-3 mAb to H. pylori-infected AGS cells (Fig. S3). Hence, although the data suggest a prominent role of Gal-3 as a chemoattractant, they also implicate other molecules that could contribute to the chemotraction of THP-1 cells. Future studies should aim at utilizing expression microarrays to further characterize the mechanisms underlying the regulation of various host response events induced by receptor–ligand interactions.

CONCLUSION

Our study outlines a previously unidentified role of extracellular Gal-3 that would regulate the H. pylori adhesion and apoptosis in H. pylori-infected gastric epithelial cells. However, the resultant effect of this may inadvertently favour the persistence of bacteria in the gastric mucosal environment in the development of a chronic infection. In addition, we also propose that Gal-3 may play the role of chemoattractant to THP-1 monocytes during H. pylori infection. These findings taken together may contribute to our better understanding of how Gal-3 acts as a modulator under pathological conditions.

REFERENCES


