BACTERIAL PATHOGENICITY

Binding of human plasminogen and lactoferrin by *Helicobacter pylori* coccoid forms

M. M. KHIN, M. RINGNÉR*, P. ALELJUNG*, T. WADSTRÖM* and B. HO

Department of Microbiology, National University of Singapore, Lower Kent Ridge Road, Singapore 0511 and
*Department of Medical Microbiology, University of Lund, Solvegatan 23 S-223 62, Lund, Sweden

The interactions between *Helicobacter pylori* spiral and coccoid forms, extracellular matrix (ECM) and plasma proteins were studied in an ¹²⁵I-labelled protein assay. The range of binding of collagen V, plasminogen, human lactoferrin (HLf) and vitronectin to coccoid forms of *H. pylori* NCTC 11637 was 26–48%. In contrast, binding of radiolabelled fibronectin and collagen types I and III was low (3–8%). The coccoid forms of 14 strains of *H. pylori* showed significant HLf binding (median 26%). With plasminogen, no significant difference was found between binding to the coccoid (median = 13%) and spiral (median = 12%) forms, of 13 of the 14 strains of *H. pylori* tested; the exception was strain NCTC 11637. ¹²⁵I-plasminogen showed a dose-dependent binding to both the coccoid and spiral forms. Plasminogen binding to both forms was specific; the binding was inhibited by non-labelled plasminogen, plasmin, lysine, EACA (epsilon-aminocaproic acid) but not by fetuin or various carbohydrates. Similarly, HLf binding was found to be specific and was inhibited by non-labelled HLf and BLf. The coccoid forms showed either similar or enhanced ECM binding capabilities compared with the spiral forms. As the binding of ECM proteins may be an important mechanism of tissue adhesion for various pathogenic bacteria, the coccoid differentiated form of *H. pylori* can be considered as an infective form in the pathogenesis of helicobacter infection and type B gastritis.

Introduction

Several bacterial pathogens express surface proteins with affinity for components of the mammalian extracellular cellular matrix (ECM) [1, 2]. ECM components can serve as targets for bacterial adhesion. Specific attachment of bacteria to host tissues is an initial step in the development of several infections. The adhesiveness of ECM components promotes bacterial colonisation and invasion of subepithelial tissues [1]. Some of the ECM glycoproteins that have been shown to interact with different micro-organisms are fibronectin, various collagens, vitronectin, laminin, thrombospondin, bone sialoprotein II, heparan sulphate and heparin [2].

It has been reported that *Helicobacter pylori* spiral

forms interact with ECM proteins such as laminin, vitronectin, plasminogen and collagen types I and IV [3–6]. Plasminogen is a 91-kDa single-chain glycoprotein present in blood and in many tissue fluids. It is enzymically inactive and is activated to plasmin by activators such as urokinase (u-PA) or tissue-type plasminogen activator (t-PA), and is involved in fibrinolysis [7–10]. Binding of plasminogen is not limited to *H. pylori* [5], but has also been reported in pathogenic gram-positive cocci and gram-negative bacilli [7, 9–12].

Lactoferrin is an iron-binding glycoprotein present both intracellularly in polymorphonuclear leucocytes (PMNLs) and in various body secretions such as milk, saliva, tears, nasal secretions and intestinal secretions [13, 14]. Lactoferrin is released from PMNLs in response to cytokine stimulation and gram-negative bacterial infections [15]. It is also reported that virulence and gastric colonisation are accomplished through an iron acquisition mechanism by the human lactoferrin (HLf)-receptor system of *H. pylori* [16].

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Corresponding author: Dr B. Ho.

It has been proposed that interactions of *H. pylori* and ECM proteins represent a mechanism of adherence to gastric tissues, thereby playing an important role in the development of chronic type B gastritis and peptic ulcer disease [5]. The source of infection and mode of transmission of *H. pylori* infection is not well understood. However, previous findings that coccoid forms of *H. pylori* contain cell-surface haemagglutinins and heparan sulphate binding proteins have suggested the survival of these forms in subcellular tissues [17] and also stimulated speculation about their role in transmission and as a possible cause of recurrence of gastroduodenal ulcers [18]. This study aimed to examine the interactions of ECM and plasma proteins with *H. pylori* coccoid and spiral forms and to compare the binding of proteins to *H. pylori* coccoid and spiral forms.

Materials and methods

Bacterial strains and spiral forms of H. pylori

Fourteen *H. pylori* strains were studied. These comprised two reference strains (NCTC 11637 and CCUG 17875); one clinical isolate from the USA (strain 6); 10 clinical isolates from Sweden; and strain 19106, a kind gift from F. Megraud (University of Bordeaux, France). The bacteria were grown on GAB-Camp agar supplemented with horse blood in a humidified incubator (Assab, Stockholm, Sweden) at 37°C in air with CO₂ 5% for 3 days, to produce homogeneous spiral forms. The spiral nature was observed by phase contrast microscopy (Nikon Microphot-FXA, Japan).

Preparation of coccoid forms

To obtain the coccoid forms, *H. pylori* NCTC 11637 was grown in 600 ml of Brain Heart Infusion Broth (Gibco BRL, Paisley), supplemented with yeast extract (Oxoid) 0.4% and horse serum (Gibco BRL) 10% in a 1-L glass fermenter vessel (Biolab, Braun, Germany). Temperature was maintained at 37°C with a water bath. Sterile CO₂ was added twice a day for 5 s each time. Dissolved oxygen was maintained by a feedback control of the stirrer speed of c. 40 rpm. The culture was incubated for 63 days to provide homogeneous coccoid forms as described by Vijayakumari [19]. In contrast, the coccoid forms of the other 13 isolates were induced in nutritionally deprived 1 × HAM's 12 medium (Flow Laboratories, USA) with fetal calf serum 10%. The coccoid forms appeared homogeneous in every field of wet smears when examined by phase contrast microscopy (Nikon Microphot-FXA). When inoculated on to moist chocolate agar and incubated in a humidified incubator (Forma Scientific, USA) at 37°C with CO₂ 5%, the broth containing coccoid forms did not show any growth after incubation for 12 days. The coccoid form has been known as viable but non-culturable [18, 20].

The cells were washed twice in PBS (pH 7.2), centrifuged at 1000 *g* for 20 min and resuspended in PBS to obtain 1 × 10⁹ cells/ml. Spiral and coccoid forms were enumerated in triplicate with a Neubauer haemocytometer counting chamber and phase contrast microscopy (Nikon, Microphot-FXA).

Chemicals

The chemicals used in this study were purchased from Sigma unless otherwise stated. Human vitronectin (Bional Ltd, Tartu, Estonia), vitrogen (Celtrix Laboratories, Sweden), human and bovine lactoferrin (HLf and BLf), human plasminogen, collagen types I, II, III and V, transferrin, bovine serum albumin (BSA), human albumin, fetuin, fibrinogen, heparin, glucose, lactose, maltose, mannose, fucose, plasmin, lysine and epsilon aminocaproic acid (EACA) were used in this study. Fibronectin was purchased from Bional Ltd.

Binding assay

Vitronectin, HLf, BLf, plasminogen, collagen, transferrin, vitrogen, BSA and human albumin were labelled with ¹²⁵Iodine by a modification of the Chloramine-T method with Iodobeads [21]. The specific activity of the respective proteins ranged from 5 × 10⁵ to 1 × 10⁶ cpm/μg. The binding assay was performed according to the method of Paulsson and Wadstrom [22]. Briefly, 50 μl of radiolabelled protein (3 × 10⁴ cpm) in PBS (pH 7.2) with BSA 1% were incubated with 100 μl of bacterial cell (10⁸ spiral or coccoid forms) suspension for 1 h at room temperature. A 100-μl volume of PBS incubated with 50 μl of ¹²⁵I-labelled protein was always included in the assay as a negative control. The reaction was stopped with ice-cold PBS containing Tween 20 0.1% and the suspension was centrifuged at 1000 *g* for 20 min. The supernate was aspirated and the activity in the pellet was counted in a 1260-Multigamma counter (LKB-Wallac, Turku, Finland). The binding was calculated as the percentage of the total amount of ¹²⁵I protein added to the bacteria. The χ² test was used for statistical analysis.

Inhibition assay

Plasminogen and HLf inhibition assays were performed by pre-incubating 100 μl of NCTC 11637 suspension (10⁸ spiral or coccoid forms) with 100 μl of each inhibitor for 1 h at room temperature. The inhibitors used in these assays were (/ml) 100 μg each of plasmin, lysine, EACA, fetuin, fibrinogen, HLf and BLf; heparin 500 IU; carbohydrates at 0.1 M; and unlabelled plasminogen or HLf in the range 1–100 μg. Subsequently, radiolabelled plasminogen or HLf was added and the binding assay was performed as described above.

Results

Binding of ECM and plasma proteins to H. pylori NCTC 11637

¹²⁵I-labelled vitronectin bound to the coccoid and spiral forms of NCTC 11637 at high levels of 48 and 51%, respectively, while collagen V showed an intermediate level of binding (26 and 21%, respectively) (Table 1). The binding of collagen types III and I, and fibronectin to both forms was low (range 3–8%). Collagen type II, transferrin, vitrogen, BSA and human albumin showed no binding to the coccoid or spiral forms. Interestingly, HLF and plasminogen bound to the spiral forms at only 10 and 16% compared with the binding capacity of 31 and 30%, respectively, to *H. pylori* coccoid forms (Table 1).

Binding of HLF and plasminogen to H. pylori

With the exception of strains 52 and CCUG 17875, the coccoid forms demonstrated a high HLF-binding capacity (median 26%, range 9–43%) that differed significantly ($p < 0.01$) from the spiral forms (median 11%, range 8–18%) (Fig. 1a). HLF showed insignificant

differences in binding capabilities to the spiral forms of the 14 strains of *H. pylori*. With plasminogen, only the NCTC 11637 coccoid forms showed 30% binding, which differed greatly as compared to 16% to its corresponding spiral forms (Fig. 1b). However, coccoid forms of the other 13 *H. pylori* strains showed no difference ($p > 0.05$) in binding of plasminogen (median 13%, range 10–19%) as compared to the spiral forms (median 12%, range 9–18%).

Specificity of plasminogen and HLF binding

Binding of ¹²⁵I-plasminogen to *H. pylori* coccoid and spiral forms was specific, as non-labelled plasminogen, plasmin, lysine and EACA inhibited the binding at high level (Fig. 2a and Table 2). The other glycoproteins and carbohydrates inhibited plasminogen binding in the range 2–23% (Table 2). Fig. 2b shows that binding of ¹²⁵I-HLF to *H. pylori* was also specific.

Discussion

The studies showed that various ECM and plasma proteins such as vitronectin, HLF, plasminogen, fibronectin, collagen types I, III and V bound to *H. pylori* NCTC 11637 coccoid forms in the range 3–48%. Many bacterial pathogens have the ability to bind to ECM proteins necessary for microbial colonisation of various host tissues [1, 2]. Both coccoid and spiral forms showed no significant difference in binding of collagen V, a major constituent of ECM proteins. The binding of spiral forms of *H. pylori* to other ECM proteins has been reported to provide an important tissue adhesion mechanism in chronic *H. pylori* infection [17]. Similarly, the coccoid forms were able to bind to these proteins, indicating that this adherence mechanism may also be present in the coccoid forms. It is possible that the surface-protein interaction with ECM by both the spiral and coccoid forms of *H. pylori* may play a vital role in gastric colonisation.

Table 1. Binding of ECM and plasma proteins by *H. pylori* NCTC 11637 coccoid and spiral forms

¹²⁵ I-labelled protein	Mean percentage binding (SEM) to	
	Coccoid forms	Spiral forms
Vitronectin	48 (0.64)	51 (0.32)
Human lactoferrin	31 (0.18)	10 (0.87)
Plasminogen	30 (1.45)	16 (1.03)
Collagen V	26 (1.22)	21 (0.61)
Fibronectin	8 (0.21)	7 (0.60)
Collagen I	8 (0.53)	6 (0.78)
Collagen III	3 (0.14)	3 (0.14)
Collagen II	1 (0.11)	1 (0.04)
Transferrin	0.9 (0.09)	0.7 (0.08)
Vitrogen	0.8 (0.03)	1 (0.06)
BSA	0.6 (0.02)	0.8 (0.07)
Human albumin	0.4 (0.01)	0.5 (0.02)

Table 2. Inhibition of plasminogen and lactoferrin binding to *H. pylori* NCTC 11637 coccoid and spiral forms

Inhibitor	Percentage inhibition by plasminogen binding (SEM) to		Percentage inhibition by lactoferrin binding (SEM) to	
	Coccoid forms	Spiral forms	Coccoid forms	Spiral forms
Plasmin	67 (0.50)	58 (0.21)	20 (1.13)	21 (0.64)
Lysin	56 (1.41)	45 (1.56)	NT	NT
EACA	83 (1.16)	69 (1.77)	NT	NT
BLf	20 (1.06)	23 (0.87)	58 (2.34)	70 (1.84)
HLf	13 (0.14)	19 (0.71)	73 (2.27)	68 (1.63)
Fetuin	7 (1.73)	10 (1.06)	3 (0.21)	5 (0.39)
Heparin	3 (0.14)	6 (0.06)	2 (0.17)	2 (0.14)
Fibrinogen	2 (0.11)	2 (0.07)	7 (0.25)	3 (0.39)
Glucose	16 (0.85)	9 (0.49)	12 (0.35)	11 (0.32)
Maltose	10 (0.28)	11 (0.99)	8 (0.43)	7 (0.71)
Lactose	7 (0.35)	8 (0.57)	14 (0.14)	16 (0.21)
Mannose	7 (0.11)	7 (0.35)	20 (0.82)	12 (0.50)
Fucose	3 (0.23)	6 (0.21)	17 (0.60)	11 (0.56)

NT, not tested.

Fig. 1. a, Binding of ^{125}I -HLf, expressed as a percentage of the total amount of ^{125}I -HLf added, to coccoid (■) and spiral (□) forms of 14 *H. pylori* strains. **b**, Binding of ^{125}I -plasminogen, expressed as a percentage of the total amount of ^{125}I -plasminogen added, to coccoid (■) and spiral (□) forms of 14 *H. pylori* strains. The *H. pylori* strains shown in **a** and **b** are as follows: 1, NCTC 11637; 2, strain 25; 3, strain 1139; 4, strain 52; 5, strain 54; 6, strain 33; 7, strain 66; 8, strain 915; 9, strain 6; 10, strain 32; 11, strain 253; 12, CCUG 17875; 13, strain 12225; 14, strain 19106.

Binding of HLf to the coccoid forms of *H. pylori* was found to be significantly higher than to spiral forms, with the exception of strains 52 and 17875 (Table 1 and Fig. 1a). HLf is an iron-binding glycoprotein

present in significant amounts in human stomach tissues with superficial or atrophic gastritis. Studies of various bacterial pathogens have shown that the ability to acquire iron *in vivo* is an important microbial

Fig. 2. a, Inhibition of plasminogen binding to coccoid (●) and spiral (▲) forms of *H. pylori* NCTC 11637 by 1–100 μg of non-labelled plasminogen. b, Inhibition of HLF binding to coccoid (●) and spiral (▲) forms of *H. pylori* NCTC 11637 by 1–100 μg of non-labelled HLF.

virulence factor [13, 23–24]. It has been reported that the iron acquisition system of *H. pylori* by the HLF receptor system may play a major role in the virulence of *H. pylori* infection [16]. In this study, *H. pylori* coccoid forms were found to bind strongly to HLF, indicating that the coccoid forms may also use an iron acquisition mechanism similar to the spiral forms in expressing their pathogenic potential, as proposed by Husson *et al.* [16]. Lactoferrin binding to *H. pylori* coccoid as well as spiral forms was observed to be specific, as the binding was inhibited by non-labelled HLF and BLf (Table 2 and Fig. 2b).

With the exception of strain NCTC 11637, plasminogen bound to the coccoid and spiral forms of 13 strains of *H. pylori* to similar extents (Table 1 and Fig. 1b). The finding that plasminogen bound more efficiently to the coccoid forms than the spiral forms of strain NCTC 11637 only suggests a possibility that the growth medium used may be associated with the adherence characteristics. It has been reported that pathogenic bacteria binding to plasminogen would result in the formation of surface-bound plasminogen on the bacteria [11]. This surface-bound plasminogen can be converted to an active enzyme (plasmin), either by locally synthesised tissue-type plasminogen activator (t-PA) or t-PA present in blood. The activation could provide a mechanism for bacteria to adopt surface-bound proteolytic activity that allows the micro-organisms to penetrate into surrounding tissues resulting in modulation of the microbial tissue tropism [11]. It is possible that *H. pylori* coccoid forms may also carry surface-bound plasminogen to enhance plasmin formation for spreading in the subepithelial environment.

In-vitro studies with *H. pylori* have reported that the spiral forms can change into coccoid forms after incubation for several days [25, 26]. Moreover, the retention of cell surface haemagglutinins and heparan sulphate-binding proteins by coccoid forms has suggested their survival in epithelial and subepithelial tissues. It has been proposed that long-term survival of the coccoid forms is important in patients with peptic ulcers healed by H₂ blockers, proton pump inhibitors or sucralphate [17], suggesting their possible role in recurrence and transmission of *H. pylori* infection. Furthermore, it has been stated that binding of HLF and plasminogen to the micro-organisms is important for virulence [11, 16]. As compared to the spiral forms, the present study shows that the coccoid forms, which are the differential forms of *H. pylori*, still possess similar specific binding receptors for plasminogen. They also have higher binding ability for HLF, suggesting a possible role in pathogenicity.

The binding of plasminogen to *H. pylori* coccoid and spiral forms was found to be specific (Fig. 2a and Table 2), as the binding was inhibited by non-labelled plasminogen and its analogues, plasmin, lysine and

EACA (Fig. 2a and Table 2). Plasminogen was reported to bind to the receptors on eukaryotic cells by a mechanism involving the lysine-binding sites [7–8, 11]. Ullberg *et al.* [8] reported that EACA (the lysine analogue) completely eliminated plasminogen uptake, suggesting that the lysine binding sites on the plasminogen molecule are involved in receptor-ligand interactions. Furthermore, surface-associated plasmin was reported to be protected against plasmin inactivators such as alpha-2-antiplasmin and alpha-2-macroglobulin [8]. The coccoid forms of *H. pylori* might prevent surface-bound plasminogen from inactivation by the several plasminogen inactivators studied, indicating their possible involvement in pathogenesis of helicobacter infection.

This study illustrates the binding of some ECM and plasma proteins to differentiated forms of *H. pylori*. Considering the importance of interactions between several ECM proteins and pathogenic bacteria, these findings suggest that coccoid forms play a role in the pathogenesis of *H. pylori* infection.

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