In vivo expression of the 25-kDa laminin-binding protein of *Helicobacter pylori*

Anthony P. Moran a,*, Samantha A. Broaders a, Anna Rapa b, Giuseppina Oderda b

a Laboratory of Molecular Biochemistry, Department of Microbiology, National University of Ireland, Galway, University Road, Galway, Ireland

b Department of Paediatrics, University of Piemonte Orientale, Novara, Italy

Received 12 May 2004; received in revised form 8 September 2004; accepted 20 September 2004

First published online 19 October 2004

Abstract

The gastroduodenal pathogen *Helicobacter pylori* has been shown to inhibit the interaction between the extracellular matrix protein laminin and its receptor on gastric epithelial cells, potentially contributing to a loss of mucosal integrity. As a 25-kDa outer membrane protein of *H. pylori* in association with the bacterial lipopolysaccharides (LPS) mediates attachment to laminin, the aim of this study was to determine whether the 25-kDa protein is produced by *H. pylori* in infected hosts. We examined the immune response to the 25-kDa laminin binding protein in 12 paediatric patients; samples from a *H. pylori*-negative healthy adult were used as controls. In immunoblotting, antibodies to a 25-kDa protein were found in the serum and saliva of *H. pylori*-positive individuals only, and using the positive sera and saliva, laminin binding to the 25-kDa protein was inhibited. Thus, the 25-kDa laminin-binding protein is produced by *H. pylori* in infected hosts.

Keywords: *Helicobacter pylori*; Laminin; Outer membrane protein; Adhesin; Immune response

1. Introduction

*Helicobacter pylori* is one of the commonest human gastrointestinal pathogens, infecting almost 50% of the human population [1]. The bacterium is the causal agent of active chronic gastritis, is associated with peptic ulcer disease, and produces an increased risk for the development of gastric adenocarcinoma and primary gastric lymphoma. A number of epidemiological studies have demonstrated that once *H. pylori* infection is acquired, it is rarely cleared and persists for the life of the individual [2].

Laminin is a complex, noncollagenous glycoprotein. By its formation of networks with type IV collagen, entactin/nidogen, and heparan sulphate proteoglycans, it is important for the structure of the basement membrane [3]. Strains of *H. pylori* have been reported to bind to laminin and also to type IV collagen in a specific and saturable manner [4–6]. *H. pylori* has been shown to interfere with the interaction between laminin and its receptor (an integrin) on gastric epithelial cells [7]. It has been demonstrated previously that a phosphorylated structure in the core oligosaccharide mediates the interaction of lipopolysaccharide (LPS) of a haemagglutinating *H. pylori* strain with laminin, whereas in contrast, a conserved non-phosphorylated structure in the core oligosaccharide mediates the interaction of a poorly haemagglutinating strain in vitro [8]. Moreover, a 25-kDa protein in the outer membrane of the bacterium mediates attachment, in association with the LPS, by both haemagglutinating and poorly haemagglutinating *H. pylori* strains [9]. Arising from observations in a
preliminary serological study of *H. pylori*-positive paediatric patients using a commercial kit that a 25-kDa band was observed in immunoblotting [10], the aim of the present study was to determine whether the laminin-binding 25-kDa protein is produced by *H. pylori* in infected human hosts, i.e., under in vivo conditions. In particular, serum and saliva samples were obtained from *H. pylori* infected and non-infected paediatric patients and the reaction of antibodies with the 25-kDa laminin-binding protein was investigated.

2. Materials and methods

2.1. Patients and specimens

Samples were obtained from twelve patients, including seven female and five male patients, attending the Department of Paediatrics, University of Turin, Italy. The mean age of the group was 9.9 years, and the age range was between 5 and 16 years. Also, samples obtained from a healthy adult volunteer were used as a negative control throughout the study.

Biopsy specimens, from the antrum, were recovered from the patients by endoscopy which was carried out under sedation and using an Olympus GIF-10 fibreoptic endoscope. On the morning of the endoscopy a blood sample and 1 ml of saliva were taken from each patient and immediately stored at −20 °C. In addition, a 13C-urea breath test was performed on each patient by giving 50 mg of 13C-urea with a solution of 10% polycose to the patient, the breath was collected at the baseline and after 30 min, and subsequently analysed with a mass spectrometer.

2.2. Analysis of patient specimens

A portion of the antral biopsy was subjected to a rapid urease test (CP-test, Gist-Brocades, The Netherlands). Another portion was inoculated onto blood agar plates containing Skirrow’s selective supplement (Oxoid Ltd., Basingstoke, UK) and was incubated for 5 days at 37 °C in a microaerobic atmosphere (5% O2, 10% CO2 and 5% N2) [11]. *H. pylori* colonies were confirmed by Gram-staining, catalase test and demonstration of urease activity [11]. Finally, another biopsy portion was fixed with Bouin’s solution, then sectioned, stained with both Giemsa and haematoxylin and eosin stains, and examined for the presence of *H. pylori* and signs of inflammation. Gastritis was defined according to the Sydney System. Antibodies (IgG) against *H. pylori* were measured in patient sera by a qualitative enzyme-linked immunosorbent assay (ELISA) using a commercially available kit (HM-CAP™, Sigma, St. Louis, MO, USA) according to the manufacturer’s instructions. Sera and saliva samples were screened for *H. pylori*-specific antigens using a commercial immunoblotting kit (Helori-WB, Eurospital, Trieste, Italy). Briefly, serum samples were tested at a 1:50 dilution and saliva samples were either undiluted or used at various dilutions from 1:1.25 to 1:4 [10]. *H. pylori* infection was defined as being present if culture alone or a combination of three of the four other tests (i.e., rapid urease test, histopathology, 13C-urea breath test, and serology) was positive.

2.3. Bacterial strains and culture conditions

Two *H. pylori* strains, one a haemagglutinating strain (NCTC 11637) and the other a poorly haemagglutinating strain (NCTC 11638), were obtained from the National Collection of Type Cultures, Colindale, UK. Stock cultures were maintained at −70 °C in 15% (v/v) glycerol–Trypticase soy broth (BBL Microbiology Systems, Cockeysville, MD, USA). *H. pylori* strains were grown routinely on blood agar under microaerobic conditions at 37 °C for 48 h. These strains had been used in previous studies characterising the interaction of *H. pylori* with laminin [8,9].

2.4. Isolation of outer membranes

Bacterial cells of *H. pylori* NCTC 11637 and NCTC 11638 were washed with and suspended in 10 mM Tris–HCl, pH 7.5, and sonicated 30 times for 30 s intervals on ice. The resulting suspension was treated with DNase II (Sigma) and RNase A (Sigma) as described previously [12], and the cell debris was removed by centrifugation (1000g for 15 min at 4 °C). Phenylmethylsulfonyl fluoride (1 mM) was added to the suspension and the whole envelope fraction was collected by centrifugation (100,000g for 1 h at 4 °C). Inner and outer membranes were separated in sucrose gradients by the method of Osborn et al. [13]. Fractions were removed, washed three times with 10 mM Tris–HCl (pH 7.5), and assayed for protein content and NADH oxidase activity [13]. The fraction containing the least NADH oxidase activity was the crude outer membrane preparation. Subsequently, this preparation was treated with 0.5% (w/v) sodium lauryl sarcosine as described previously [14] to solubilise any contaminating cytoplasmic proteins. The sarcosyl-insoluble membrane fraction (outer membrane) was collected by centrifugation (40,000g for 30 min at 4 °C), washed three times with high-grade water, aliquoted, and stored at −20 °C until used. The purity of outer membrane preparations was confirmed by the absence of NADH oxidase activity.

2.5. Production of anti-laminin polyclonal antisera

Laminin was obtained from AMS Biotechnology (Stockholm, Sweden). Specific antiserum to laminin
was produced as described previously [12]. Briefly, lambs were given intradermal injections at several sites with purified laminin (1 mg) in 10 mM Tris–buffered saline pH 7.4 (TBS) mixed with an equal volume of complete Freund’s adjuvant. Booster injections were given every third week using the same antigen concentration, but without adjuvant. When an adequate antibody titre was obtained, the lambs were exsanguinated and the serum was collected and stored at −20 °C. Preimmune sera from the same animals were used as controls.

2.6. Western blotting with H. pylori-positive and -negative serum and saliva samples

To determine whether antibodies against the H. pylori 25-kDa protein were present in control and patient sera and saliva samples, Western blotting studies were performed. H. pylori cells were harvested, resuspended in phosphate-buffered saline (PBS) to give 0.3 absorbance units at 600 nm. From these preparations, whole-cell (WC) extracts were prepared using the method of Hitchcock and Brown [15]. These WC extracts and outer membrane preparations of NCTC 11637 and NCTC 11638 were subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using a constant current of 35 mA, a stacking gel of 4% (w/v) acrylamide, and a separating gel of 10% (w/v) acrylamide [9]. Separated proteins were electrotransferred to nitrocellulose membranes (Hybond C extra, Amersham, England) by the method of Sankaran et al. [14]. After the transfer, the non-specific binding sites were blocked with a blocking solution (dried skimmed milk, 5 g; TBS, 100 ml) at room temperature for 1 h with gentle agitation. The membranes were washed for 5 min in washing buffer composed of TBS containing 0.001% (v/v) Tween 20. The patient and control sera and saliva samples (primary antibodies) were incubated with the membranes at 37 °C for 2 h. The membranes were washed three times for 5 min prior to incubation with the diluted secondary antibody (1:1000), horseradish peroxidase (HRP)-conjugated goat anti-human IgG (Sigma, St. Louis, MO, USA), at 37 °C for 1 h. Immunoreactants were visualised using a horseradish peroxidase substrate development kit (Bio-Rad).

2.7. Inhibition of laminin binding by sera and saliva antibodies

To confirm that H. pylori-positive sera and saliva samples not only bound a H. pylori 25-kDa outer membrane protein (OMP) but also bound that associated with laminin binding, further immunoblotting studies were performed. As described above, WC extracts and OMPs were electrotransferred to nitrocellulose membranes, and then blocked with TBS containing 1% (w/v) bovine serum albumin (BSA) [12]. Firstly, to detect the laminin-binding protein, the nitrocellulose membranes with separated proteins were incubated at 20 °C for 3 h with 300 µg of laminin in TBS containing 0.03% (v/v) Tween 20 (TBS-Tween) [9]. After incubation the membranes were washed with TBS-Tween and reactive bands were visualised as described above with lamb antiserum to laminin as the primary antibody (1:1000) and anti-lamb IgG HRP conjugate (Dakopatts, Copenhagen, Denmark) as the secondary antibody (1:3000) [6,9]. Preimmune sera from the same animals were used for control experiments. Another set of control experiments were conducted, in which the electrotransferred proteins on the nitrocellulose membrane were not incubated with laminin, anti-laminin lamb antisera or both, but the rest of the above procedure was carried out. To confirm the specificity of interaction of laminin with the 25-kDa OMP, electrotransferred OMPs on nitrocellulose membranes were pre-incubated with 300 µg of 3-sialyllactose (Oxford GlycoSystems, Abingdon, UK) before incubation with laminin [9].

Secondly, inhibition of laminin binding by patient and control sera and saliva samples was assessed whereby the sera and saliva samples were incubated with nitrocellulose membranes of electrotransferred WC extracts and OMPs of H. pylori NCTC 11637 and NCTC 11638 at 37 °C for 2 h. Subsequently, this membrane was overlaid with laminin and immunoreactants were visualised as described above.

Thirdly, the 25-kDa laminin-binding protein of H. pylori NCTC 11637 was purified by ammonium sulphate precipitation and laminin-Sepharose affinity chromatography [16] and used as a positive control in the inhibition experiments above.

3. Results

3.1. Western blotting of positive and negative control sera with separated OMPs and WC extracts

Data regarding paediatric patient characteristics, H. pylori infection status and serological reactivity against the 25-kDa protein are presented in Table 1. Of the H. pylori-infected paediatric patients, three of eight (37.5%) had active gastritis, another four had non-active gastritis (50%) and one (12.5%) was diagnosed with intestinal metaplasia. Serum and saliva samples were available from 11 patients, but only a serum sample was available from patient F. In addition, serum and saliva samples were available from a healthy adult who was screened for infection and shown to be H. pylori-negative by culture, histopathology, 13C-urea breath test, and serology.

Similar to the findings of a previous study [9], the outer membrane preparations of haemagglutinating and non-haemagglutinating H. pylori strains, NCTC 11637
and 11638, respectively, contained a 25-kDa protein (Fig. 1(A)) to which laminin bound (Fig. 1(B)). Representative of the immunoblotting results obtained, serum from patient H (*H. pylori*-positive) reacted with a 25-kDa protein in both WC and OMP preparations of *H. pylori* NCTC 11637 (Fig. 2(A)) and also with similar preparations of NCTC 11638 (data not shown). In contrast, serum from patient K (*H. pylori*-negative) did not react with WC and OMP preparations of NCTC 11637 (Fig. 2(B)), nor did the *H. pylori*-negative adult serum (Fig. 2(C)). Likewise, no reaction was observed with WC and OMP preparations of NCTC 11638 by serum from patient K or the adult serum. In a similar manner, saliva from patient H, but not from patient K nor the adult subject, reacted with WC and OMP preparations of NCTC 11637 and NCTC 11638 (data not shown). Collectively, the sera and saliva samples of all *H. pylori*-infected patients, but not uninfected individuals, reacted with a 25-kDa OMP of both the haemagglutinating and non-haemagglutinating strains examined (Table 1).

### Table 1

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th><em>H. pylori</em> infection</th>
<th>Pathology</th>
<th>Anti-25-kDa protein reactivity&lt;sub&gt;b&lt;/sub&gt;</th>
<th>Serum</th>
<th>Saliva</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>16</td>
<td>+</td>
<td>Active gastritis</td>
<td>+++&lt;sup&gt;c&lt;/sup&gt;</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>12</td>
<td>+</td>
<td>Intestinal metaplasia</td>
<td>++</td>
<td></td>
<td>++</td>
</tr>
<tr>
<td>C</td>
<td>9</td>
<td>+</td>
<td>Active gastritis</td>
<td>+++</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>D</td>
<td>10</td>
<td>+</td>
<td>Non-active gastritis</td>
<td>+++</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>E</td>
<td>13</td>
<td>+</td>
<td>Non-active gastritis</td>
<td>+++</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>F</td>
<td>10</td>
<td>+</td>
<td>Non-active gastritis</td>
<td>+++</td>
<td></td>
<td>n.d.&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>G</td>
<td>11</td>
<td>+</td>
<td>Active gastritis</td>
<td>+++</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>H</td>
<td>9</td>
<td>+</td>
<td>Non-active gastritis</td>
<td>+++</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>I</td>
<td>8</td>
<td>–</td>
<td>Absent</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>J</td>
<td>8</td>
<td>–</td>
<td>Absent</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>K</td>
<td>8</td>
<td>–</td>
<td>Absent</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>L</td>
<td>5</td>
<td>–</td>
<td>Absent</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

<sup>a</sup> Results of *H. pylori* infection: +, positive; –, negative.

<sup>b</sup> Results were graded visually: +++, very strong positive; ++, strong positive; –, negative.

<sup>c</sup> n.d., not determined as saliva sample was not available.

### 3.2. Inhibition experiments

Serum and saliva samples from patient H and patient K were chosen as positive and negative controls from the paediatric patient group and subjected to further testing. Firstly, immunoblotting with laminin and electrotransferred OMPs yielded an immunoreactive band indicative of laminin binding to the 25-kDa OMP protein of *H. pylori* NCTC 11637 (Fig. 3(B)), whereas in control experiments where laminin was not added (Fig. 3(C)) or non-immune serum was used instead of anti-laminin primary antibody (Fig. 3(D)), no immunoreactivity was observed. Also, no immunoreactivity was observed in other control experiments where anti-laminin antiserum or both this antiserum and laminin were absent. Moreover, the specificity of the interaction of laminin with the 25-kDa OMP was confirmed by complete inhibition of binding with 3-sialyllactose (data not shown). Serum from patient H inhibited the binding of laminin to the 25-kDa OMP (Fig. 3(E)), whereas serum from patient K (Fig. 3(F)) or serum from the *H. pylori*-negative adult did not inhibit the binding interaction since immuoreactive bands were observed. Identical results to those above were obtained when OMP preparations of NCTC 11638 or purified 25-kDa laminin-...
Previously, we showed in vitro that laminin binding by \textit{H. pylori} is mediated by LPS and a 25-kDa OMP acting as a 3-sialyllactose-specific, lectin-like adhesin \cite{8,9}. However, whether this 25-kDa OMP was produced in vivo remained unanswered.

In the present study, saliva and serum samples from 12 paediatric patients were examined for the presence of antibodies against the 25-kDa laminin-binding protein. This study population was chosen since a preliminary serological study of \textit{H. pylori}-infected paediatric patients, using a commercial immunoblotting kit, had shown the reactivity of samples with a 25-kDa band \cite{10}. Of the eight \textit{H. pylori}-positive individuals, both sera and saliva samples reacted with a 25-kDa OMP of \textit{H. pylori} haemagglutinating and non-haemagglutinating strains. These \textit{H. pylori}-positive individuals displayed a range of pathologies; three had chronic active gastritis (i.e., with polymorphonuclear granulocytes infiltrating the gastric mucosa), four had chronic non-active gastritis (i.e., with polymorph infiltration), and one had intestinal metaplasia. No recognition of the 25-kDa OMP was observed with samples from the four remaining \textit{H. pylori}-negative paediatric patients. Likewise, serum and saliva samples of the control \textit{H. pylori}-negative adult did not react with the 25-kDa OMP. Thus, the results showed that a 25-kDa OMP of \textit{H. pylori} was produced in vivo; however, to confirm that this was the laminin-binding protein inhibition studies were undertaken. These studies clearly showed that sera and saliva samples from \textit{H. pylori}-positive individuals inhibited laminin binding to this 25-kDa OMP, whereas \textit{H. pylori}-negative samples did not, thereby confirming expression of the laminin-binding protein in vivo.

Emphasising the importance of the experimental approach, in early preliminary investigations attempting to identify the \textit{H. pylori} protein adhesin interacting with laminin, one group attributed the interaction to a conserved afimbrial 19.6-kDa protein with weak haemagglutinating activity \cite{19}. However, subsequent studies showed that this protein was a cytosolic iron-binding protein and that binding to laminin was due to non-specific hydrophobic interactions \cite{20}. Likewise, a 20-kDa protein (HpaA) with haemagglutinating activity and binding specificity for 3-sialyllactose, the same isomeric form as bound by the 25-kDa laminin-binding protein \cite{21} was reported, but later shown to be a lipoprotein located in the cytoplasmic fraction of the cell \cite{22}. Importantly, during immunoblotting in the present study, conditions were employed to minimise any non-specific hydrophobic interactions \cite{9,20} and, as in our previous investigation \cite{9}, the specificity of the interaction of laminin with the 25-kDa OMP was confirmed by complete inhibition of binding with 3-sialyllactose. Also, our stringently purified outer membrane preparations when analysed by SDS–PAGE and Coomassie blue staining indicated the absence of HpaA \cite{22} and other known hydrophobic cytoplasmic proteins \cite{23}. Furthermore,
the purified 25-kDa laminin-binding protein gave identical results to those obtained with the outer membrane preparations.

The observation in the present study that the sera and saliva samples of all infected individuals contained antibodies to the 25-kDa laminin-binding protein is consistent with the importance of this protein in pathogenesis. The ability of H. pylori to bind laminin [4–6] may in part explain the association of the bacterium with intercellular junctions [9]. Moreover, as the oligosaccharides of laminin are important in the extracellular matrix-cell adhesion mechanism [17,18], and the interaction of integrins on gastric epithelial cells with laminin is inhibited by H. pylori LPS [7], the disruption of epithelial cell-basement membrane interaction after H. pylori binding laminin could partly explain the development of lost mucosal integrity during H. pylori infection, including contributing to disruption of tight junctions and development of gastric epithelium leakiness. Importantly, as well, antibodies against the H. pylori LPS core structures, that have been implicated in laminin binding, were detected in H. pylori-infected individuals [24] thereby indicating that these structures, along with the 25-kDa protein, are also available for interaction with laminin in vivo.

Evidence indicates that in most populations H. pylori infection is commonly acquired in childhood. Also, it has been suggested that H. pylori infection can influence growth which emphasises the clinical relevance of early detection of H. pylori infection in childhood [25]. In addition to serum antibody levels, specific salivary IgG antibodies in adults and children have already been applied for diagnosis of H. pylori infection [25,26]. In general, salivary antibody testing has the advantages of being non-invasive which is of particular relevance for testing of children, saliva samples do not require any particular handling or storage, and such sampling compared with obtaining blood samples could reduce the risk of blood-borne infection. As shown in this study, both sera and saliva samples of H. pylori-infected individuals contained anti-25-kDa protein antibodies. Moreover, in a preliminary immunoblotting study with paediatric sera and saliva samples, in addition to the presence of a 120-kDa band (CagA protein) as a marker for infection with H. pylori, detection of a 25-kDa protein band was found useful [10]. Therefore, in the formulation of future serological assays for paediatric patients, particularly salivary antibody-based systems, the inclusion of the 25-kDa laminin-binding protein should be evaluated.

Interestingly, Voland et al. [27] found three low-molecular-weight proteins, one of which was a 32-kDa neuraminylactose-binding haemagglutinin precursor, that reacted with sera from H. pylori-infected patients. Although the 25-kDa laminin-binding protein is 3-sialyllactose-specific, the relationship of the 25-kDa laminin-binding protein to the latter haemagglutinin is unclear, especially since a number of sialic-acid specific haemagglutinins have been described in H. pylori. Also, in contrast to the present study, salivary antibodies were not evaluated. Based on the observation that the immune response against the low-molecular-weight antigens decreased by ≥70% at 5-months post-eradication, Voland et al. [26] suggested that the immune response against these antigens might provide a serological assessment not only for H. pylori infection, but also for eradication. Whether the immune response against the laminin-binding protein could be of value for eradication assessment is a further question to be addressed.

Acknowledgements

This study was supported by grants from the Irish Higher Education Authority (PRTL-1) under the National Development Plan and the Irish Health Research Board.

References


