Haemophilus influenzae and airway inflammation in chronic bronchitis
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Interaction of clinical isolates of nonencapsulated *Haemophilus influenzae* with mammalian extracellular matrix proteins

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Abstract

The adherence of clinical isolates of nonencapsulated *Haemophilus influenzae* strains from patients with chronic bronchitis to distinct immobilized extracellular matrix components was determined. With selected strains the induction of plasmin formation by these isolates was studied. The strains could be divided into two groups: strains that showed a very high level of adherence to laminin and type I collagen, as well as adhesion to fibronectin and strains that showed only a moderate level of adhesion to laminin and a low level of adhesion to fibronectin. Plasmin formation was demonstrated for three out of eight isolates. Persisting and nonpersisting strains did not differ quantitatively or qualitatively with respect to the level of adhesiveness to the distinct matrix proteins and in their ability to induce plasmin formation.

Introduction

Chronic *Haemophilus influenzae* infections are prominent in the airways of patients with chronic bronchitis and chronic obstructive pulmonary disease (COPD) [1,2]. Persistence of *H. influenzae* strains for many months and reinfections with ‘new’, unrelated strains are frequently observed in these patients [3,4]. These infections are associated with a local inflammatory response [2,3,5], epithelial damage and exposure of the underlying extracellular matrix (ECM) [6-8]. In patients infected with *H. influenzae* accumulation of bacteria was observed between and underneath epithelial cells [9,10]. Read et al. [11] have demonstrated in a model consisting of resected human nasal turbinates that *H. influenzae* not only adhered to epithelial cells after damaging these cells, but also to the exposed extracellular matrix.

Many pathogenic bacteria, including *H. influenzae*, express surface proteins that enable them to adhere to the ECM and its components via specific interactions [12-14]. We have previously demonstrated that also distinct nonencapsulated *H. influenzae* strains were able to adhere to the ECM and ECM components, like laminin, fibronectin and various collagens [15].

In addition to adherence to ECM components, nonencapsulated *H. influenzae* strains can bind plasminogen [16] that can be activated by tissue-type plasminogen activator (t-PA) [15,17]. Plasmin generated on nonencapsulated *H. influenzae* plasminogen receptors was demonstrated to degrade ECM components [15] and to potentiate
bacterial penetration through a basement membrane preparation reconstituted on membrane filters [15].

These results suggest that binding of *H. influenzae* to the ECM and degradation of the ECM may promote local bacterial persistence and spread through subepithelial layers. Spread of *H. influenzae* into lung tissue was observed in explants of lungs from persistently infected patients with cystic fibrosis and COPD undergoing lung transplantation [10].

Since these results suggest that binding to the ECM might be an important step in the pathogenesis of persisting infections in patients with chronic bronchitis and COPD, we determined whether clinical isolates of *H. influenzae* from chronic bronchitis patients adhered to ECM components. In addition, the induction of plasmin formation by these strains was analyzed.

**Materials and Methods**

**Characteristics of patients and bacterial strains**

Nonencapsulated *H. influenzae* were isolated from sputum samples of patients with chronic bronchitis during a monthly follow-up. All patients had a history of chronic bronchitis as defined by a productive cough on most days of the year for at least 3 months of the year during two or more consecutive years [18]. In addition, all patients had culture proven chronic or recurrent infections with *H. influenzae*. *H. influenzae* was identified by its dependence for growth factors X and V and its inability to convert δ-aminolevulanic acid to porphyrins [19]. The strains were characterized phenotypically by major outer membrane protein (MOMP) subtyping as described previously [20]. In addition, genotypic characterization was performed by randomly amplified polymorphic DNA (RAPD) analysis with the primers ERIC1 and ERIC2 as described before [21]. The RAPD patterns obtained were shown to be strain specific. Strains (*n* = 11) with a distinct MOMP pattern were considered persisting since they were cultured for at least 6 months from various, consecutive sputum samples from a patient. Strains (*n* = 8) were considered nonpersisting since they were cultured only on one occasion during a monthly follow-up for at least 6 months and, in addition, phenotypically and genotypically different strains were cultured before and after that period. All clinical isolates were used within two passages of the original isolation.
Chapter 4

Culture conditions
The strains were cultured overnight at 37°C on chocolate agar plates in a humidified atmosphere supplemented with 5% CO₂. Bacteria from the agar plates were inoculated into brain-heart infusion broth supplemented with 1% IsoVitalex (BBL Microbiology Systems, Cockeysville, MD, USA) and 40 mg/L hemin (Sigma, St. Louis, MO, USA). After overnight culturing, bacteria were collected by centrifugation (10,000 x g, 10 min) and washed twice with PBS, pH 7.1 [15].

Adherence tests
Bacterial adherence to ECM proteins coated on glass was tested as described before [15,22]. Bacteria were used at a concentration of 5 x 10⁷ to 10⁹ cells/mL. The surface concentration, 2.5 pmol of type I, IV, and V collagens (Sigma), laminin (Upstate Biotechnology, Lake Placid, NY, USA), and human plasma fibronectin (Collaborative Research) was achieved as described [22]. Control proteins fetuin and albumin (BSA; Sigma) were coated on glass from a solution containing 25 μg of the protein ml⁻¹. Adherent bacteria were visualized as described before [15] after staining with methylene blue, in an Olympus (Olympus Optical Co., Hamburg, Germany) microscope equipped with a charged couple device camera (4912-5000: Cohu, San Diego, CA, USA) and images were digitized using an LG-3 (Scion, Frederick, MD, USA) scientific frame grabber and Macintosh 7100/80 MHz computer using the public domain NIH Image 1.55 program (written by W. Rasband, National Institute of Health, Bethesda, MD, USA). The number of bacteria in 20 microscopic fields of 1.6x10⁴ μm² was determined using density slicing.

Plasminogen activation
Kinetic measurements of plasminogen activation were performed as described before [17,23,24]. The bacterial densities were 2x10⁸ and 4x10⁸ ml⁻¹; plasminogen (Biopool, Umeå, Sweden) was tested at 20 μg ml⁻¹, t-PA (Biopool) was tested at 50 ng ml⁻¹, the chromogenic plasmin substrate S-2251 (Kabivitrum, Stockholm, Sweden) was tested at 0.45 mM and the plasminogen activation inhibitor e-aminocaproic acid (EACA; Sigma, St. Louis, MO, USA) was tested at 1 mM, in a test volume of 200 μl. After incubation 90 min at 37°C, the OD₄₀₅ of the supernatant was measured. For these experiments we used eight isolates, four persisting (5581, 5612, 7087 and A850048) and four nonpersisting (0664, 4564, 7087 and 8754) strains that were tested in two independent experiments.
Results

**Adherence of persisting and nonpersisting H. influenzae to ECM components**

Adherence of the different bacterial isolates to isolated components of the mammalian ECM coated on glass, studied in two independent experiments, is summarized in Table 1. Adherence to fetuin (a highly glycosylated protein) and BSA (a nonglycosylated protein) were used as controls. None of the strains adhered to BSA, whereas some strains showed a very low level of adherence (− to +) to fetuin. Adherence to ECM components, but not to control proteins was shown to be concentration dependent.

With respect to adherence to the matrix components two distinct adherence profiles were observed. The first group showed strong adhesion (2 + to 5 +) to laminin and type I collagen and adhesion (1 + to 3 +) to fibronectin. Furthermore, most of the strains in this group adhered also to type V and to a lesser extent type IV collagen. Compared to this group, the second group showed a weaker adhesion to both laminin (1 + to 3 +) and fibronectin (− to 2 +) and no or only a very weak adhesion to the collagens (− to 1 +). Four of 11 persisting strains (A860516, 6888, 6653 and 2890) and four of eight nonpersisting strains (5170, 8754, 7087 and 0664) showed characteristics of the first type (Table 1). Persisting and nonpersisting strains did not differ quantitatively or qualitatively in the way they adhered to the specific ECM components.

**Plasminogen activation**

We examined whether clinical isolates of *H. influenzae* were able to induce plasminogen activation. Therefore, we studied the plasmin formation by four persisting and four nonpersisting isolates. Plasmin formation could only be demonstrated by the persisting strains 5581 and A850048 and the nonpersisting strain 4564. They all belonged to the group of strains weakly adhering to laminin. Plasmin formation could not be demonstrated with any of the strains that showed a strong adherence to laminin and collagen I (0664, 7087 and 8754). These data indicate that distinct *H. influenzae* strains isolated from sputum samples of chronic bronchitis patients may express plasminogen receptors on their surface independent of whether the bacteria are persisting or nonpersisting.
Table 1. Adherence of *H. influenzae* isolates (n = 19; 5x10^6 bacteria ml^{-1}) to ECM components and controls

<table>
<thead>
<tr>
<th>Strain</th>
<th>P/NP*</th>
<th>Laminin</th>
<th>FN</th>
<th>Col I</th>
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Discussion

In this study we have demonstrated that part of the chronic bronchitis isolates adhered strongly to immobilized extracellular matrix proteins, indicating that binding of *H. influenzae* to exposed ECM components may contribute to bacterial adherence, an essential step in the pathogenesis of airway infection in patients with chronic bronchitis and COPD.

The high level of adherence to various ECM components, especially laminin and collagen I, indicate that these components are important recognition sites for *H. influenzae*. Laminin is part of the basement membrane, which forms under physiological conditions a gel-like structure resembling in structure the lamina densa
zone of the basement membranes [25]. Collagen I is a fibrillar collagen. In this respect, *H. influenzae* adherence resembles the adherence of *Yersinia enterocolitica* through YadA, a critical surface protein involved in invasion [26]. It is tempting to speculate that *H. influenzae* has a similar mechanism to adhere to and penetrate epithelial cell layers before these bacteria find a subepithelial niche to persist. Since, however, part of the persisting strains showed only a low level of adherence to the matrix proteins tested, our results indicate that this type of adherence may facilitate penetration of *H. influenzae* into subepithelial tissues, but is not a prerequisite for local persistence of bacteria in chronic bronchitis and COPD patients. On the other hand, we cannot exclude that the nonadhering (persisting) strains may bind to other receptors of the ECM not tested. Candidate adhesins of *H. influenzae* are fimbriae, high molecular mass proteins (HMW) 1 and 2, Hia protein and Hap protein. These adhesins mediate also binding to epithelial cells [13,14]. Since we have previously demonstrated that two nonfimbriated nonencapsulated *H. influenzae* strains adhered efficiently to ECM proteins, like laminin, fibronectin and various collagens, fimbriae are not likely to be involved [15]. HMW1 protein binds to cellular glycosaminoglycans [27] and might therefore be a candidate adhesin. However, strain A850081 not adhering to ECM components (Table 1) expressed HMW1, adhered to epithelial cells through a HMW1 specific mechanism and was demonstrated to be positive in PCR for the *hmw1* gene (unpublished observation). Since *H. influenzae* also expresses the other adhesins, adherence to matrix proteins is one of the mechanisms *H. influenzae* is using to attach to the airway mucosa.

Our studies on plasmin formation by clinical *H. influenzae* isolates indicate that distinct strains may express plasminogen receptors on their surface. Interestingly, these strains did show only a weak adherence to the matrix proteins. Plasmin formation could not be demonstrated by any of the strains adhering strongly to the matrix components. These results indicate that adherence to ECM and binding of plasminogen are independent phenotypes, or that one excludes the other. Plasmin formation was not restricted to persisting or nonpersisting isolates. Previously, we have shown that plasminogen binding increased the penetration potential of *H. influenzae* in all cases [15], thereby suggesting that plasminogen binding and subsequent plasmin formation are required for invasion of bacteria in the underlying tissues.

In conclusion, we demonstrated that clinical isolates of *H. influenzae* vary in the level of adherence to immobilized extracellular matrix components and in their ability to induce plasminogen activation. Plasminogen activation was observed only for nonadhering strains. Persisting strains did not differ from nonpersisting strains, indicating
that other mechanisms must be operative for the local persistence of *H. influenzae* observed in patients with chronic bronchitis and COPD.

**References**


