MICROBIAL PATHOGENICITY

Promotion of *Escherichia coli* adherence to rubber slices by adsorbed fibronectin

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Summary. Biomaterial-associated infections are a problem in the use of endoprosthetic materials in the palliative treatment of malignant obstructive jaundice. Fibronectin has been reported to mediate adherence of bacteria to host tissue and biomaterials. Adsorption of fibronectin to rubber—representing material used for biliary drainage—and subsequent adherence of *Escherichia coli* strain PSS1 and *E. coli* strain NG7C (which binds to immobilised fibronectin) were investigated. Quantitative adsorption of fibronectin to rubber slices was studied with ¹²⁵I-labelled, purified human plasma fibronectin. In buffer solutions, fibronectin showed a high affinity for rubber slices. Adherence of the *E. coli* strains to uncoated rubber slices was similar and was significantly inhibited by the presence of plasma components and bile. Adherence of *E. coli* PSS1 to fibronectin-coated slices was poor. In contrast, *E. coli* NG7C adhered efficiently to coated slices in proportion to the amount of adsorbed fibronectin; adherence was not reduced by the presence of albumin or bile, or the fibronectin-binding ligands gelatin, heparin and fibrinogen. However, pre-digestion of coated slices with trypsin significantly reduced adherence.

Introduction

Since biliary endoprosthesis (stent) insertion was introduced by Soehendra in the late 1970s,¹ it has become an effective treatment for internal biliary decompression in both malignant and benign biliary obstruction.² ³ However, the procedure is frequently complicated by blockage of the endoprostheses with biliary sludge, impairing function and necessitating replacement.⁴ ⁵ Although various factors, including the role of bacteria and endoprosthetic material, have been investigated,⁷ ⁹ the mechanisms underlying the endoprosthesis blockage are not fully understood. Fibronectin, a host-derived glycoprotein present in bile¹⁰ ¹¹ as well as in plasma, has been reported to be a mediator of bacterial adherence to implants *in vivo*.¹² However, the affinity of fibronectin for biliary endoprosthetic materials and its role in infection associated with biliary implants have yet to be established.

In the present study, the interactions between biliary endoprosthetic materials—represented by rubber slices—fibronectin and *Escherichia coli* strains were investigated in an in-vitro model.

Materials and methods

Chemicals and materials

Fibrinogen (bovine, fraction I, type I), purified trypsin and purified human albumin were obtained from Sigma (St Louis, MO, USA), [methyl-³H]-thymidine and Na¹²⁵I from Amersham Laboratories (Buckinghamshire), scintillation fluid (Scintran*) from BDH Chemicals Ltd (Poole), iodination reagent (Iodobeads*) from Pierce Chemical Co. (Rockford, IL, USA), Sepharose 4B, gelatin-Sepharose and arginine-Sepharose from Pharmacia Chemicals Co. (Uppsala, Sweden), gelatin and activated latex beads (0·8 μm diameter) from Difco (Detroit, MI, USA), rubber T-tubes from Rüsch AG (Kernen, Germany) and heparin from Løvens Kemiske Fabrik (Ballerup, Denmark).

Fibronectin was purified from pooled human plasma by the method of Vuento and Vaheri.¹³ The concentration of purified fibronectin was determined from absorbance values at 280 nm with a 1-cm path length cuvette on the basis of an $E_{1\text{mg/mL}}$ value of 1·28. The purity of fibronectin was confirmed by SDS-PAGE. Fibronectin-depleted plasma was obtained by passing plasma through Sepharose 4B and gelatin-Sepharose.¹⁴ Fibronectin was labelled with ¹²⁵I by the method of Markwell.¹⁴ Radioactivity of the labelled fibronectin was 400000 (SEM 5000) cpm/10 μl (n = 12).
Rubber slices (1 cm² each) were prepared from rubber T-tubes, briefly rinsed with ethanol (99.5%) followed by three rinses in sterile water and then sterilised by heating at 120°C for 30 min.

Bile ducts of Sprague-Dawley rats were ligated and transected, pooled rat bile was obtained by puncture of the dilated bile ducts. Human bile was obtained during surgery from patients undergoing cholecystectomy, sterilised by filtration (Millipore; pore size 0.45 μm), and stored at -20°C until used.

**Bacterial strains**

Two *E. coli* strains were studied. *E. coli* NG7C expresses high binding of soluble and surface immobilised fibronectin, whereas *E. coli* PSS1 produces P fimbriae and expresses binding of soluble fibronectin but not of immobilised fibronectin. Binding of immobilised fibronectin to cells of both strains was determined by the method of Naidu *et al.* Organisms were stored in TSB Broth (Difco) containing glycerol 10% at -20°C and subcultured on blood agar twice before experiments.

**Radiolabelling of bacterial strains**

Overnight cultures (2 × 10⁸ cfu) of each *E. coli* strain in colonisation factor antigen (CFA) broth were inoculated in 1 ml of CFA broth containing [methyl-³H]-thymidine 100 μl and incubated at 37°C for 4 h to a density of 3 × 10⁹ cfu/ml. In preliminary experiments, bacteria took up more radioactivity in CFA broth than in other broths over this time period. Unbound radioactivity was removed by centrifugation twice at 3000 g for 10 min and the labelled cells were resuspended in 2–3 ml of 0.15 M NaCl.

**Adsorption of fibronectin on to rubber slices**

Different concentrations of fibronectin (5–160 μg/ml) in phosphate buffered saline, PBS, pH 7.2 were prepared from a stock solution (1:27 or 2:4 mg/ml) containing (8 × 10⁸)-10⁶ cpm of ¹³¹I-labelled fibronectin. Adsorption of fibronectin on rubber slices was estimated by the method of Vaudaux *et al.* Each rubber slice was incubated with 1 ml of fibronectin solution for 60 min at 37°C with agitation; unbound radio-labelled fibronectin in the fluid was then determined. The slices were washed three times in 2 ml of PBS at 20°C, transferred into clean polystyrene tubes and radioactivity was counted in a Gamma counter (model no. 2772, LKB-WALLAC, Turku, Finland). For each fibronectin concentration tested, the recovery of total radioactivity was calculated by adding up the radioactivity of unbound fibronectin, the washing fluid, the rubber slice and the fibronectin adsorbed on the wall of the polystyrene tubes.

Fibronectin adsorption from plasma or from bile to rubber slices was tested by supplementing fibronectin-depleted plasma or pooled rat bile with fibronectin (5–160 μg/ml) containing radiolabelled fibronectin as a tracer. The adsorption assay was then performed as described above.

To analyse the adsorption kinetics of fibronectin to rubber slices, a model of saturation kinetics, derived from the Langmuir equation, was used. In this model, the ratio of unbound/bound molecules (C/Q) is plotted against the concentration of unbound molecules (C); in a monolayer, this will result in a straight line relationship if all binding sites are independent and have identical affinities for the adsorbate molecules. The adsorption parameters, including the maximal number of adsorption sites (N) and the affinity constant (K), can be calculated from the slope and intercept of this line according to the formula:

\[
\frac{C}{Q} = \frac{1}{KN} + \frac{C}{N}
\]

**Bacterial adherence assays**

Adherence assays were performed at 37°C in PBS supplemented with 1 mM Ca²⁺ and 0.5 mM Mg²⁺ (pH 7.2). When human albumin, rat serum or plasma was included in the adherence medium, the protein concentration was adjusted to 5 mg/ml measured by the BioRad® protein assay (BioRad Laboratories GmbH, München, Germany). Concentrations of bacteria were adjusted to 10⁶ cfu/ml. Adherence to fibronectin-coated rubber slices was studied after incubation of slices for 60 min at 37°C in unlabelled fibronectin (5–160 μg/ml) as described above; the adherence medium was PBS containing 1 mM Ca²⁺ and 0.5 mM Mg²⁺, supplemented with human albumin 5 mg/ml where stated. Native or fibronectin-coated rubber slices were incubated with labelled *E. coli* (10⁷ cfu/ml) for 60 min at 37°C. The slices were then transferred to new tubes, washed three times in 2 ml of 0.15 M NaCl, immersed in scintillation fluid and the radioactivity was counted in a liquid scintillation counter (model no. LS 1800, Beckman Instruments, Fullerton, CA, USA). The cpm were multiplied by the ratio of cfu/cpm (average 72, SEM 20 cfu/cpm, n = 24), to give the number of adherent bacteria.

Rubber slices coated with fibronectin by incubation with fibronectin (160 μg/ml) as described above were used in further studies of *E. coli* NG7C adherence: (i) the fibronectin-coated rubber slices were treated for 20 min at 37°C with purified trypsin 10 μg/ml of PBS (pH 7.2) before the adherence assay; (ii) the fibronectin-binding ligands gelatin, heparin and fibrinogen were added to the adherence medium at concentrations of 1000 μg/ml, 500 U/ml and 500 μg/ml respectively.

**Statistical methods**

Values are presented as mean and SEM. Linear regression and unpaired Student's t test were used for statistical analysis.
E. coli ADHERENCE AND FIBRONECTIN

Fig. 1. Fibronectin adsorption to rubber slices as a function of unbound fibronectin in the presence of PBS (□—□), defibrinated plasma (△—△) or bile (○—○).

Fig. 2. Saturation kinetics of adsorption of fibronectin to rubber slices.

Results

Langmuir adsorption isotherms of fibronectin bound to rubber slices

Fibronectin binding to rubber slices proved to be dose-dependent in unsupplemented PBS (fig. 1); results correlated well with the Langmuir adsorption model (fig. 2; coefficient of correlation = 0.887). The derived number of adsorption sites was $1.07 \times 10^{14}$ molecules/cm$^2$. The affinity constant was $2.23 \times 10^{-15}$ ml/molecule. Fibronectin binding was poor in the presence of fibronectin-depleted plasma or bile.

Adherence of E. coli to rubber slices

Adherence of E. coli NG7C and E. coli PSS1 to uncoated rubber slices under various conditions is shown in table 1; the presence of human albumin, rat plasma, rat serum, rat bile or human bile inhibited adherence of both strains by > 90% in comparison with controls. With fibronectin-coated slices, adherence of E. coli NG7C (which binds to immobilised fibronectin) was similar to that observed with uncoated material; however, adherence of E. coli PSS1 was reduced and was significantly less than for E. coli NG7C ($p < 0.001$). In contrast to results with uncoated slices, adherence of E. coli NG7C to fibronectin-coated slices was not reduced in the presence of albumin or bile.

Fig. 3 shows the dose-dependent adherence of E. coli NG7C to fibronectin-coated slices in albumin-supplemented PBS. The pattern of adherence of E. coli
Table I. Influence of albumin, serum, plasma and bile on the adherence of *E. coli* strains NG7C and PSS1 to rubber slices

<table>
<thead>
<tr>
<th>Addition to adherence medium*</th>
<th>Mean (SEM, n = 6) number of <em>E. coli</em> (cfu × 10^5) adherent to rubber slices</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Uncoated slices</td>
</tr>
<tr>
<td></td>
<td>Fibronectin-coated slices†</td>
</tr>
<tr>
<td></td>
<td>PSS1</td>
</tr>
<tr>
<td>None</td>
<td>1280 (130)</td>
</tr>
<tr>
<td>Human albumin</td>
<td>44 (2)†</td>
</tr>
<tr>
<td>Pooled rat plasma</td>
<td>64 (3.4)†</td>
</tr>
<tr>
<td>Pooled rat serum</td>
<td>50 (3.4)†</td>
</tr>
<tr>
<td>Pooled rat bile</td>
<td>62 (4.3)†</td>
</tr>
<tr>
<td>Human bile</td>
<td>59 (3.0)†</td>
</tr>
</tbody>
</table>

ND, not determined.
* PBS supplemented with divalent cations.
† Rubber slices coated by incubating in fibronectin 160 μg/ml.
‡ p < 0.001 compared with supplemented PBS alone.
§ p < 0.001 compared with adherence of *E. coli* PSS1 to fibronectin-coated slices.

NG7C followed closely that of fibronectin adsorption to rubber slices (as shown in fig. 1) and, as expected from the fibronectin adsorption studies, adherence of the organism was poor when fibronectin adsorption on rubber slices was performed in the presence of fibronectin-depleted plasma or bile.

The specificity of *E. coli* NG7C binding to fibronectin-coated rubber slices was studied. Trypsin treatment of fibronectin-coated rubber slices resulted in a significant reduction in adherence of *E. coli* NG7C (p < 0.001, fig. 4). Binding of *E. coli* NG7C to fibronectin-coated slices was not affected by the presence of heparin or gelatin in the adherence medium. However, fibrinogen promoted adherence of *E. coli* NG7C cells to both uncoated slices and fibronectin-coated slices (table II).

Discussion

Some bacterial pathogens adhere strongly to foreign materials and often become embedded in a thick matrix of exopolysaccharides, leading to permanent colonisation of the implant. Such problems can occur with biliary stents, which, after a number of months in situ, become covered by biofilm containing bacteria which eventually cause stent blockage. In another study, we demonstrated that, after implan-
Fig. 4. Effect of trypsin (10 µg/ml) treatment of fibronectin-coated rubber slices on the binding of E. coli NG7C.

Table II. Effects of fibronectin-binding ligands on the adherence of E. coli NG7C to rubber slices

<table>
<thead>
<tr>
<th>Addition to adherence medium*</th>
<th>Mean (SEM, n = 4) number of adherent E. coli (cfu x 10⁶)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>Fibronectin-coated slices† Uncoated slices‡</td>
</tr>
<tr>
<td>None</td>
<td>780 (21)        &lt; 10‡</td>
</tr>
<tr>
<td>Gelatin (1000 µg/ml)</td>
<td>770 (45)        &lt; 10‡</td>
</tr>
<tr>
<td>Heparin (500 U/ml)</td>
<td>850 (36)        &lt; 10‡</td>
</tr>
<tr>
<td>Fibrinogen (500 µg/ml)</td>
<td>920 (50)§       88 (21)§</td>
</tr>
</tbody>
</table>

* PBS supplemented with divalent cations and human albumin (5 mg/ml).
† Rubber slices coated by incubating in fibronectin 160 µg/ml.
‡ p < 0.001 compared to fibronectin-coated slices.
§ p < 0.01 compared to supplemented PBS alone.

However, in the presence of whole plasma or bile, fibronectin adsorption on to the rubber slices was severely inhibited. This inhibition may be due to common amphiphilic surface properties or to competitive adsorption. Bile contains amphiphilic substances which may induce micelle formation and block hydrophobic binding sites. Some proteins, including albumin, secretory IgA, azo-dye metabolite binding protein, fibronectin and complement components, have been detected in human bile. Fibronectin promotes cholesterol crystallisation in bile which further changes the physical and chemical properties of bile. In the presence of other proteins, fibronectin is adsorbed differently to surfaces quantitatively as well as qualitatively, and potential bacteria-binding domains may not be exposed.

Interestingly, even though rat bile and human bile had different inhibitory effects on E. coli adherence to native rubber slices, neither inhibited adherence to those pre-coated with fibronectin. These findings suggest that, in vivo, when implants are covered by host-derived proteins such as fibronectin and glycosaminoglycans, bile does not prevent bacterial adherence and subsequent development of infection.

A further finding in the present study was the quantitative relationship between the adherence of E. coli NG7C cells and the amount of fibronectin deposited on the slices. Bacterial adherence may be an additional, sensitive, quantitative assay of one of the multiple biological activities of fibronectin. Although little is known about the binding sites on fibronectin for E. coli NG7C, the lack of interference of adherence of E. coli NG7C to fibronectin-coated rubber slices by gelatin, heparin or fibrinogen at high concentrations indicates that there are binding sites distinct from those for these ligands.

There are two major types of fibronectin; fibronectin soluble in plasma and other extracellular fluids, and fibronectin associated with the surface of cells as a constituent of the extracellular matrix (ECM) of various connective tissues. Our studies in vitro on the adsorption of fibronectin to biliary materials (represented by rubber slices), provides only a simplified view of the in-vivo environment of foreign bodies. In the presence of fibronectin-depleted serum, some biomaterials adsorbed more fibronectin when they were pre-coated with proteins such as denatured
collagen, and, furthermore, the adsorbed fibronectin exerted more active biological effects. Similar effects were observed in collagen, and, furthermore, the adsorbed fibronectin contributed to the biological activity of the foreign body adsorbing protein which then enhances the adsorption and biological effects of fibronectin. A further difference in the in-vivo environment is the longer time course, allowing changes of configuration of adsorbed protein, and, in turn, resulting in different bacteria-binding domains of fibronectin and other adsorbed proteins becoming exposed.

Two independent pathways may be involved in the deposition of fibronectin on the surface of foreign bodies in vivo, i.e., trapping of soluble fibronectin from the extracellular fluids bathing the implants, and endogenous synthesis and deposition of cell-associated fibronectin by cells colonising around foreign bodies. The first pathway is probably more important in foreign body infection in the biliary tract. Further in-vivo investigation is needed to explain the possible role of fibronectin and other bile proteins in implant-associated infection in the biliary tract.

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References


