Vitronectin may mediate staphylococcal adhesion to polymer surfaces in perfusing human cerebrospinal fluid

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Introduction

In neurosurgery, prosthetic devices are frequently used for the management of clinical conditions implying impairment of the cerebrospinal fluid (CSF) circulation, e.g., hydrocephalus. The CSF may require continuous diversion (shunts), or devices may be used intermittently for sampling, drug administration (reservoir) or drainage. The prosthetic devices may be internalised (e.g., shunts, reservoirs) or externalised. Externalised devices are most commonly used in the treatment of infected shunts, and they may also be used for intracranial pressure (ICP) monitoring, or for the temporary diversion of CSF from an obstructed ventricular system.

The main complications following CSF shunt surgery are shunt dysfunction and infection; almost as soon as shunts were instituted in clinical practice, reports of infections appeared [1]. The mean rate of infection following primary shunt insertion in reported studies since 1980 is c. 13% [2–4]. Shunt revisions are associated with a higher incidence of infection. The highest rate of infection (c. 40%) has been reported in premature neonates with intraventricular haemorrhage and external ventricular drains (EVD) [5].

Bacteria belonging to the normal skin flora, such as coagulase-negative staphylococci (CNS) and Propionibacterium acnes are the dominating aetiological agents.

Once bacterial pathogens enter the central nervous system host defence mechanisms are inadequate to control the infection. Immunoglobulins and complement components are largely excluded from the CSF, and the opsonic activity of CSF is far below that of...
serum even in the presence of meningitis [6, 7]. The low immunoglobulin concentration concomitant with the local complement deficiency contribute to the regional host deficiency in the CSF during infection. Proteins are also largely excluded from the CSF by the blood–CSF barrier. The total CSF protein concentration in normal adult lumbar CSF is 0.5 g/L, and the CSF:serum ratio of albumin is 1:200. The protein content of lumbar CSF is c. 1.6 times higher than ventricular CSF, and cisternal CSF c. 1.2 times higher. The protein content of CSF may be elevated by subarachnoidal haemorrhage or traumatic lumbar puncture.

Within seconds of the insertion of a biomaterial, host proteins adsorb to its surface. The adsorbed proteins undergo changes of configuration after adsorption to a varying extent, depending on the physicochemical properties of the polymer surface and on the properties of the protein [8–15]. Hence, the surface exposure of different domains of host proteins can differ significantly during the first 24 h. Microbes may colonise the biomaterial surface by receptor-specific interaction with domains on the adsorbed proteins, by hydrophobic interaction or by nonspecific mechanisms [16]. The importance of microbial colonisation on the external versus the internal surface has not been established, but colonisation of the external surface of intravascular catheters has been shown to be correlated to the development of catheter-related septicemia [17]. Studies on protein adsorption from CSF to different polymer surfaces identified fibronectin (Fn), fibrinogen (Fg), vitronectin (Vn), plasminogen, complement factor C3c and some human serum albumin (HSA) (F. Lundberg et al., unpublished observation). Fn and thrombospondin (TSP) have been proposed to mediate adhesion of Staphylococcus aureus to polymer surfaces [18, 19].

Different approaches have been made to prevent infections, such as antibiotic prophylaxis [20], and coating or blending of the polymer tubing with an antibiotic or disinfectant [21–25]. Polymethylmethacrylate intra-ocular lenses, exposed to a body fluid similar to CSF, have been heparinised by endpoint attachment [26]. The clinical experience of the use of these lenses has been highly promising, with a low rate of infections and other complications [26].

The purpose of the present study was to analyse adhesion of CNS strains, isolated from EVDs and shunts in the CSF, and from the CSF of patients with no biomaterial, to a polymer, polyvinylchloride (PVC), with and without heparin. The PVC tubes were perfused with pooled CSF for 1 h, or with CSF plus plasma 10% for 24 h, before inoculation of bacterial cells. The strains were examined for their ability to bind to Fn, Vn and heparin [27, 28], and for production of slime [29], to identify strain-specific characteristics that could predict subsequent infection.

Materials and methods

**CSF**

CSF was collected from patients with EVD with no clinical signs of central nervous system infection at the Department of Neurosurgery, University Hospital of Lund, Lund, Sweden. The CSF was pooled and tested for bacterial growth by culture on blood agar aerobically and anaerobically at 37°C for 24 h. Erythrocytes were removed by centrifugation at 2000 rpm for 10 min at 4°C. The protein concentration was determined by the BioRad protein microassay (BioRad, Richmond, CA, USA); for pooled CSF it was 0.3 g/L and for pooled CSF with plasma 10% it was 3.4 g/L. The content of Fn and Vn in pooled CSF with and without plasma 10% was assayed with rabbit antibodies to Fn or Vn and HRP-labelled swine antirabbit IgG antibodies [30]. The concentration of Vn was 29 and 30 µg/ml in CSF and CSF with plasma 10%, respectively, and that of Fn was 4.4 µg/ml in both fluids.

**Bacterial strains and culture conditions**

A total of 18 CNS strains was isolated from 15 patients. One of the patients had two episodes of shunt infection. The strains were isolated from extirpated ventriculoperitoneal (N = 9) and ventriculoatrial shunts (N = 2), and EVD (N = 4). The shunt-associated strains were isolated from shunt valves (N = 2), the ventricle part of the catheter (N = 3) and CSF (N = 6). In addition, three CNS strains isolated from the CSF of patients with no biomaterial inserts were studied. S. aureus strain ISP546 was used as a reference strain (Table 1). The strains were identified to species level by biochemical tests as described earlier [31]. The biomaterial-associated strains were identified as S. epidermidis (N = 13), S. hominis (N = 3), S. lugdunensis (N = 1) and S. capitis (N = 1). Bacterial strains were grown on blood agar (horse erythrocytes 5% v/v) for 20 h at 37°C, washed in 10 ml of 0.15 M sodium phosphate-buffered saline (PBS) pH 7.2, and resuspended in PBS at a concentration of 5 x 10⁸ cfu/ml. For adhesion assays, the suspensions were centrifuged (2600 rpm, 15 min) and resuspended in 10 ml of 0.1 M Tris-acetate buffer with 0.5 mM CaCl₂ (TA) at the same concentration.

**Binding of heparin, Fn and Vn**

Heparin binding was tested in 96-well polystyrene trays (Maxisorb, Nunc, Denmark) with endpoint-attached heparin; 100-µl volumes of bacterial suspension (5 x 10⁷ cfu/ml) were incubated on the heparin surface for 1 h at 37°C. The wells were washed three times in PBS with Tween 20 0.05%, and 100 µl of trichloroacetic acid (TCA) were added to each well. The amount of ATP was analysed in a luminometer as described earlier [32]. Tests were performed in duplicate, and values are expressed as percentage of retained
ATP in relation to total added ATP. Expression of binding of Fn and Vn was analysed in the same way. The wells were then coated with 100 μl (5 μg/ml) of Fn or Vn overnight at 4°C. Non-specific binding was blocked by incubation with bovine serum albumin (BSA) 1% in PBS for 1 h at 20°C.

**Slime production**

Slime production was analysed after growth for 18 h on sucrose agar with Congo red according to the method of Strachan et al. [29]. *S. epidermidis RP12*, which produces slime, and *S. hominis* SP2 which does not, were used as positive and negative controls [33]. Slime production was semi-quantified (0–3) where 3 denotes that the bacteria changed the colour of the agar medium to black, 2 that more than half of the agar surface was changed to black, 1 that a small part of the agar surface had changed to black or grey, and 0 denotes no slime production, i.e., no change of the colour of the medium. Tests were performed three times on different days.

**Polymers**

Polyvinyl chloride (PVC) tubings, Portex 800/000/180, with an outer diameter of 3 mm and inner of 1.5 mm were purchased from Hemex Medical, Sollentuna, Sweden. No silicone was added to the PVC during manufacture. Heparin was endpoint attached as described earlier [34]. The tubings were exposed to ethanol 70% for 10 min at 20°C, and then rinsed three times in sterile PBS (c. 200 ml). When heparinised and unheparinised PVC tubings were compared by scanning electron microscopy, the unheparinised PVC showed a rounded surface with regular ditches whereas the heparinised surface (hep-PVC) was flat and did not show any ditches. For the studies of protein adsorption and bacterial adhesion, 4-cm long pieces were introduced into a flow cell glass tube with latex fittings [30] (Fig. 1).

**Adhesion assay**

Adhesion was studied in a perfusion system where 4-cm long pieces of tubing (3-mm outer and 2-mm inner diameter) were placed in a cylindrical glass flow cell (diameter 12 mm, length 40 cm) (Fig. 1). The samples were kept at 6 cm distance from the ends of the glass tube to ensure laminar flow according to Reynold's index [35]. Test samples were perfused at a medium velocity of 0.05 m/s at 37°C.

Washed bacterial cells (5 x 10^10 cfu in 100 ml) were inoculated into the perfusion system either directly, or after perfusion of pooled CSF for 1 h, or of pooled CSF with human plasma 10% for 24 h at 37°C at a medium velocity of 0.05 m/s. After 2 h, the surface was rinsed by perfusion of TAE (200 ml, three times), and bacterial adhesion to the external and luminal surfaces was quantified by bioluminescence as described earlier [32]. The number of bacteria in each sample was calculated from a strain-specific standard curve. Quadruplicate samples were analysed and the results were expressed as mean cfu/cm² tubing and SEM.

**Inhibition of bacterial Fn and Vn binding by saturating binding sites**

Washed bacterial cell suspensions (0.3 ml) were incubated with Fn or Vn (20 μg/ml) for 1 h at 25°C. After centrifugation (10 000 rpm, 5 min) cells were resuspended in TN at the original concentration, and tested for adhesion in wells with Fn or Vn as described above. Inhibition of adhesion was expressed as any reduction in relation to the adhesion of untreated bacterial cells. Duplicate samples were analysed.

**Perfusion with antibodies to fibronectin and vitronectin to block bacterial adhesion**

To study the influence of exposure of Fn and Vn on bacterial adhesion, pooled CSF was perfused through PVC tubing with and without endpoint-attached heparin (hep-PVC) for 1 h. Thereafter, rabbit antibodies to human Fn and Vn (640 μg/ml and 320 μg/ml in PBS with BSA 1%) were applied to the surfaces for 4 h with gentle agitation. Non-specific binding was blocked by incubation in BSA 1% for 1 h. After gentle rinsing the tubing was re-inserted into the flow cell. Three strains of *S. epidermidis* (5703, 9855 and 14611), all isolated from biomaterial-associated infections, were selected for this experiment as they expressed binding of Fn and Vn to varying extents. The strains were grown to promote expression of binding of Fn, Vn and
heparin, and the tubing was exposed to the test strains for 2 h at 37°C. Quantification of bacterial adhesion was performed as described above.

Scanning electron microscopy
Pieces of PVC and hep-PVC were exposed to CSF + plasma 10% overnight, PBS with BSA 1% for 1 h and to 10⁹ cells of S. epidermidis strain 14611 for 2 h in the perfusion cell. After removal from the cell, pieces were washed twice in sodium cacodylate buffer, treated with glutaraldehyde 3% for 3 h and post-fixed in osmium tetroxide 1% with 0.1 M cacodylate buffer for 1 h. Each piece was dehydrated stepwise in ethanol at increasing concentrations (50–99%) for 5 min twice. Sputtering with gold was carried out in a SEM coating unit E5150, including a thin film monitor unit (Polaron Equipment Ltd), to a final layer of 15 nm. Examination of the whole surface of each sample was done with a Philips 515 scanning electron microscope (SEM) at 20 kV.

Statistical determinations
Student’s unpaired two-tailed t test was used when appropriate; p < 0.05 was regarded as significant.

Chemicals
Heparin was from KABI Pharmacia, Stockholm, Sweden, agar base from Oxoid, BSA from Boehringer Mannheim, Mannheim, Germany, and fresh human thrombocyte-poor plasma as purchased from the Blood Bank, University Hospital of Lund. ATP Monitoring Reagent and ATP standard were purchased from BioThema, Stockholm, Sweden. Fn was purified from human plasma according to the method of Vuento and Vaheri [36], and Vn from urea-activated human plasma according to Yatohgo [37]. Antibodies to human Fn and Vn were raised in rabbits as described earlier [30]. All salts were of analytical grade.

Results
Heparin binding
Most strains from infected biomaterials (n = 15) expressed detectable binding of heparin (1.3–21%, median 3.9%) as measured under static conditions (Fig. 2). Strains retrieved from non-biomaterial-associated sources (n = 4) also bound heparin, varying from 2.5 to 12% (median 5.4%). There was no significant difference in expression of heparin binding between the two groups.

Cells of CNS strains adhered to a varying extent (0.06 × 10⁹/cm²–13.5 × 10⁶/cm²) to PVC and hep-PVC during perfusion of CSF. The majority of strains (11 of 19) adhered in higher numbers to unheparinised PVC than to hep-PVC (Fig. 5a and b). Pre-perfusion of CSF for 1 h reduced subsequent adhesion of all but three strains, and four strains adhered at significantly higher numbers to hep-PVC than to PVC (Fig. 5a, Fig. 6a and b). Pre-perfusion of the tubings with CSF + plasma 10% overnight further reduced bacterial adhesion with seven of 19 strains (Fig. 5a, Fig. 7a and b). Compared to adhesion in the presence of PBS,
adhesion was reduced in 15 of 19 strains. All but four of 19 strains adhered to the same extent or lower to hep-PVC than to unheparinised PVC.

There was no correlation between adhesion to PVC or hep-PVC and expression of binding of Fn or Vn by the bacterial strains (Figs. 2–7). Strains expressing binding of heparin under static conditions adhered to the same extent to both heparinised and unheparinised PVC under perfusion conditions as strains which did not express heparin binding.

**Vitronectin and fibronectin binding**

The extent of binding of Vn and Fn by CNS varied, 0.1–2.3% and 0.2–2.1%, respectively (Figs. 3 and 4).
Fig. 5. Bacterial adhesion to clean unheparinised PVC (■) and hep PVC (□) under perfusion conditions. Adhesion is expressed as 10⁶ cells/cm² tube surface. Significant differences are indicated: *p < 0.05 and †p < 0.001. a, Adhesion of shunt strains as follows: 1, BD 7886; 2, BD 12213; 3, BD 6694; 4, BD 5703; 5, BD 9855; 6, BD 10260; 7, BD 14611; 8, BD 8008; 9, BD 8127; 10, BD 3338; 11, BD 9853. b, Adhesion of EVD (1–4) and non-biomaterial-associated strains (5–8) as follows: 1, BD 10282; 2, 16301; 3, BD 1897; 4, BD 1502; 5, BD 9229; 6, BD 13807; 7, BD 7743; 8, ISP 546.

The three non-biomaterial-associated CNS strains expressed binding of heparin and two of them also bound Vn and Fn. To elucidate if expression of binding of Fn or Vn mediates adhesion to PVC exposed to CSF or CSF with plasma 10%, the wells were also coated with Fn and Vn, respectively. Pre-incubation of bacteria with Vn reduced subsequent adhesion of 11 CNS strains and of *S. aureus* ISP 546, and increased adhesion of three strains (Fig. 3). Pre-incubation of bacterial cells with Fn significantly reduced adhesion of six CNS strains, and increased adhesion of one strain (Fig. 4). For biomaterial-associated strains, the reduction of binding with either Vn or Fn was significant (p = 0.0046 and p = 0.0002, respectively) whereas pretreatment with Vn or Fn did not significantly reduce subsequent binding of EVD and control strains (Figs. 3 and 4). Strains recovered from biomaterials did not express binding of Vn or Fn to a higher extent than...
**Fig. 6.** Bacterial adhesion to PVC (■) and hep-PVC (▲) under perfusion conditions. Pre-perfusion with CSF was carried out for 1 h before bacterial perfusion. Adhesion is expressed as 10⁶ cells/cm² tube surface. Significant differences are indicated: *p < 0.05, and **p < 0.001. **a.** Adhesion of shunt strains as follows: 1, BD 7886; 2, BD 12213; 3, BD 6694; 4, BD 5703; 5, BD 9855; 6, BD 10260; 7, BD 14611; 8, BD 8008; 9, BD 8127; 10, BD 3338; 11, BD 9853. **b.** Adhesion of EVD (1–4) and non-biomaterial strains (5–8) as follows: 1, BD 10282; 2, 16301; 3, BD 1897; 4, BD 1502; 5, BD 9229; 6, BD 13807; 7, BD 7743; 8, ISP 546.

non-biomaterial-associated strains. However, the number of strains in the latter group was too small to permit firm conclusions to be drawn.

**Slime production**

Two CNS strains isolated from biomaterials were strong slime producers, two were medium, five were weak producers and six did not produce slime over an 18-h period (Table 1).

Pre-incubation of tubes in the perfusion system with antibodies to human Fn or Vn

Antibodies to Vn blocked subsequent adhesion of cells of strain BD 5703 which expressed binding of both Vn
and Fn to unheparinised PVC but not to heparinised PVC (Fig. 8a), and of BD 14611 which expresses low binding of Fn and Vn, to heparinised but not to unheparinised PVC compared to pre-incubation of the system with pre-immune rabbit IgG (Fig. 8b). With strain BD 5703, the reduction was dose dependent and significant ($p = 0.04$) and with strain 14611 the reduction with heparinised PVC was significant ($p = 0.02$).

Pre-incubation of tubing in the perfusion system with antibodies to Fn or with rabbit IgG did not reduce adhesion; nor did antibodies to Vn reduce adhesion of cells of strain BD 9855, which expresses low binding of Vn and moderate binding of Fn (data not shown).

After perfusion of hep-PVC and non-heparinised PVC with cells of BD 14611 for 2 h in the presence of CSF...
Table 1. Characteristics of strains used in study

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Strain</th>
<th>Sample origin</th>
<th>Diagnosis</th>
<th>Species</th>
<th>Slime production*</th>
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VP, ventriculoperitoneal shunt; CSF, cerebrospinal fluid; VA, ventriculoatrial shunt; EVD, external ventricular drainage.

*1, weak; 2, moderate; 3, strong production (see Materials and methods).
†Strain isolated from the surface of the biomaterial.
‡Ventriculitis due to Candida parapsilosis; CNS strain interpreted as non-significant finding.
§Viral meningoencephalitis; CNS strain interpreted as non-significant finding.

Intraventricular EVD extirpated the day of discharge, no growth was found on extirpated ventricle catheter nor in CSF. The patient returned 8 days later, presenting meningitis due to the CNS strain above.

Fig. 8. Inhibition of adhesion with anti-Vn antibody (■) and pre-immune IgG (□) in increasing concentrations (320 and 640 μg/ml). PVC and hep-PVC, pre-perfused with CSF including plasma 10% for 24 h. Adhesion is expressed as 10⁶ cells/cm² tube surface: a, with strain BD 5703 to PVC tubes 53% at 320 and 36% at 640 μg/ml antibody concentrations, p = 0.00960 and p = 0.0365, respectively; b, with strain BD 14611 to hep-PVC tubes 25% at 640 μg/ml antibody concentration, p = 0.0156.

with plasma 10%, SEM showed clusters of bacterial cells on the inside of non-heparinised PVC. Fewer cells were visible on the outside of both non-heparinised PVC and hep-PVC, and only single colonies on the inside of hep-PVC. No slime was visible.

Discussion

Infections constitute a common and serious complication in the use of biomaterials in various fields of medicine. It is well known that once established, biomaterial-associated infections are almost impossible
to treat with antibiotics alone, and often require the removal of the implant [38]. Hence, it is important to prevent the initial adhesion of bacteria to the polymer. The most prevalent opinion is that bacteria colonise the biomaterial around the time of implantation. Prophylactic antibiotics have been administered to cover the time of surgery (<48 h) [20]. Alternative approaches include the incorporation of an antibiotic in the polymer or coating the biomaterial surface with an antibiotic or disinfectant [21]. Neither of these types of surface-treated biomaterials have gained widespread use, and both carry the risk of inducing allergic reactions in patients or antibiotic resistance in bacteria, or both. Heparin coating of CSF shunts reduced bacterial adhesion in vitro but low-level, surface coating in vivo has made it less promising as an alternative approach [39]. However, surface heparinisation with endpoint-attached heparin, which makes the surface more hydrophilic and confers a negative surface charge, seems to decrease bacterial colonisation of polymers exposed to ocular fluid as well as to venous blood [26, 40]. Furthermore, these surfaces are antithrombogenic and reduce complement activation [41]. Although staphylococcal strains have been shown to bind heparin under static conditions [27, 28], the results of adhesion of staphylococcal strains to immobilised heparin during perfusion do not indicate that staphylococci commonly bind to this kind of surface in vivo (Figs. 5–7).

By elucidating the pathogenesis of biomaterial-associated infections, specific measures can be taken to prevent colonisation and subsequent infection. Fn has been proposed to mediate adhesion of S. aureus to polymers in the bloodstream [16]. Studies on Escherichia coli infections of biomaterials in bile corroborate this finding, as the IgG fraction of anti-Fn serum inhibited adhesion of E. coli to biliary stents pre-perfused with bile [42]. In the present study, pre-incubation of the polymers with antibodies to Vn (but not to Fn) reduced subsequent adhesion of bacterial cells. This is interesting, as Fn has been shown to absorb to a proportionally higher extent than expected from the concentration in the protein fluid administered to some polymer surfaces [9]. Also, when bacterial cells were pre-incubated with Vn to block binding, reduced adhesion was found with more strains of CNS (11 of 19) than when cells were pre-incubated with Fn (6 of 19).

On the other hand, pre-incubation of some CNS strains with Vn or Fn increased subsequent adhesion. This may reflect binding between the glycoproteins and structures absorbed on the polymer surface. A number of specific interactions have been described, such as collagen–Fn, Fn–fibrinogen, collagen–collagen and laminin–fibronecint [43].

The present study employed a perfusion system for body fluids and compared bacterial adhesion to catheters which had not previously been exposed to CSF with adhesion to catheters that had been perfused with CSF for 1 h (Figs. 5 and 6) in an attempt to mimic the polymer surface at the time of implantation in the human body as well as to polymers which had been perfused with CSF plus human plasma 10% (Fig. 7). Some haemorrhage is inevitable during implantation, and furthermore, EVDs commonly drain bloody CSF. The protein domains exposed on these surfaces are likely to differ significantly from those on surfaces perfused with CSF for 1 h because of the postadsorptive change of configuration of Fn and Vn, and also because of the contribution of plasma proteins not normally present in CSF. It has not been elucidated if the protein content of CSF changes when a biomaterial is present. The study used CSF pooled from neurosurgical patients with EVD and without clinical signs of infection, and it is thus possible that the protein content differs from that of healthy people. It was recently shown that proteins adsorbed to a heparinised surface did not change configuration compared to proteins adsorbed to unheparinised surfaces [15]. However, only fibrinogen and HSA were analysed, proteins with no recognised heparin-binding domain.

Nine strains expressing low binding of immobilised heparin under static incubation adhered to a lower extent to the hep-PVC than to non-heparinised PVC under perfusion conditions. This may be due to low affinity binding, and hence susceptibility to shear rate-induced detachment [44, 45]. The experimental conditions where binding to immobilised heparin was analysed under static conditions differ significantly from the adhesion studies during perfusion, and cannot be used to predict subsequent adhesion to a heparinised surface introduced in perfusing body fluids.

Another approach is to identify specific virulence determinants on bacterial strains colonising, for example, the nose and forehead of the patient. The present study could not correlate expression of binding of Fn, Vn or heparin to increased adhesion to perfused polymer surfaces (Figs. 2–7). Furthermore, no correlation to development of infection was demonstrated, but the number of strains isolated from the CSF of patients without a biomaterial insert was too small to permit conclusions to be made. The finding that some strains isolated from patients without a biomaterial insert adhere to a high extent while other strains isolated from infected biomaterials adhered in very low numbers may mean that bacterial cells can adhere by binding to different host proteins, and that the affinity of binding is more important than the number of adherent cells. The number of bacterial cells introduced in the perfusion system is quite high compared to the situation in vivo and may result in saturation of the surface with bacteria and subsequently bacteria–bacteria interaction, i.e., bacterial surface aggregation. Also, surface irregularities may
cause non-specific adhesion of bacteria. Bacteria were seen adhering both as solitary cells and in clusters. Cluster formation could possibly be an artifact produced by increased surface tension at the air–water interface [46]. Bacterial adhesion to polymers in vivo may further be influenced by as yet unidentified bacterial properties.

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