MICROBIAL PATHOGENESIS

Binding of von Willebrand factor by coagulase-negative staphylococci

DAI-QING LI, FREDRIK LUNDBERG and ÅSA LJUNGH

Department of Infectious Diseases and Medical Microbiology, Lund University, Lund, Sweden

Coagulase-negative staphylococci (CNS) are the most common infectious microorganisms isolated from prosthetic devices. To determine whether von Willebrand factor (vWF) acts as an adhesin in bacterial recognition, bacterial binding of recombinant vWF (rvWF) was studied. Eleven CNS strains, belonging to S. epidermidis, S. haemolyticus and S. hominis species, bound soluble rvWF, but to a lesser extent than S. aureus. S. epidermidis strain H2-W bound 125I-labelled rvWF in a dose-dependent manner. The binding could be inhibited by unlabelled rvWF and thrombospondin, but not by fibrinogen, vitronectin or the carbohydrates N-acetylgalactosamine, D-galactose, D-glucose, and D-fucose. Pre-incubation of rvWF with type I collagen and Arg-Gly-Asp-Ser (RGDS) peptides did not inhibit binding, whereas pre-incubation of rvWF with heparin decreased binding significantly. The interaction between CNS and rvWF was sensitive to proteinase treatment of bacterial cells. CNS strains bound to immobilised rvWF an extent greater or equal to the positive control strain S. aureus Cowan I. rvWF binding structures from bacterial cell wall were detected by immunoblot. Cowan I strain had 140-, 90- and 38-kDa binding molecules. S. haemolyticus strain SM131 and S. epidermidis strain H2-W had two (120 and 60 kDa) and five (120, 90, 60, 52 and 38 kDa) binding molecules, respectively. Similar binding structures were formed when cell wall extracts from these strains were incubated with thrombospondin. These results indicate that specific ligand–receptor interaction between CNS and rvWF may contribute to bacterial adhesion and colonisation on biomaterial surfaces. Heparin-binding domains of rvWF might be the crucial regions for bacterial attachment. rvWF and thrombospondin may recognise similar molecules in staphylococcal cell wall extracts.

Introduction

Coagulase-negative staphylococci (CNS), particularly Staphylococcus epidermidis, are the most common organisms causing infections associated with foreign bodies, such as intravascular catheters, vascular grafts and cerebrospinal fluid shunt devices. Although the mechanism by which staphylococci adhere to the surface of the implanted material is still not fully understood, the interaction between bacteria and adsorbed host factors is thought to play a very important role. Once adhered to the polymer surface, bacteria proliferate and accumulate in multilayer cell clusters through intercellular adhesion and contribute to the formation of the so-called biofilm. S. aureus and CNS binding of collagen, fibronectin and fibrinogen and other proteins in serum and in extracellular matrix have been reported [1-7], but there have been few investigations as to whether von Willebrand Factor (vWF), another host protein mediating cell attachment, also provides specific information to bacteria to modulate adhesion.

Although vWF was discovered 73 years ago, its structures and gene were not determined until the 1970s [8]. It is a large multifunctional glycoprotein existing in human plasma as a series of heterogeneous homo-multimers ranging in size from c. 450 kDa to > 20 000 kDa [9]. It is required for the adhesion of platelets to sites of vascular damage, linking specific platelet membrane receptors to constituents of subendothelial connective tissue [10]. It also binds to and stabilises blood coagulation factor VIII in the circulation [11]. Most recently, Herrmann et al. tested the interaction of vWF with S. aureus. They suggested a role of vWF in the pathogenesis of intravascular S.
Materials and methods

Bacterial strains and culture conditions

A total of 11 CNS strains was tested, of which 10 strains were isolated from patients with serious graft infections, osteomyelitis and catheter-related sepsis and one strain, S. hominis SP 2, was a skin contaminant (Table 1). Three S. aureus strains and two Microccocus species were used as reference strains. Bacterial strains were grown on blood agar (horse erythrocytes 5%) for 20–22 h at 37°C except when different media were compared, washed twice in 0.1 M phosphate-buffered saline (PBS; pH 7.2) and resuspended at a final density of 10^10 cells/ml and used promptly for various binding assays.

Chemicals

Human vitronectin was purified according to Yatogho et al. [15] and human fibrinogen was purchased from Imco AB, Stockholm, Sweden. Human thrombospondin was a kind gift from Professor J. Lawler, Boston, MA, USA. All common chemicals were of analytical grade, purchased from Kebo, Spånga, Sweden. Agar bases were from LabM, Bury. ATP monitoring reagent and detachable ELISA plates from Costar, Cambridge, MA, USA. Human recombinant vWF was purchased from Immuno AG, Wien, Austria. Pronase E and proteinase K, RGDS peptides and hyaluronic acid were purchased from Sigma. Complete™ mini (proteinase inhibitor) was from Boehringer Mannheim, GmbH, Mannheim, Germany. PVDF membrane was from Micron Separations, Westborough, MA, USA. Heparin sodium salt was from Fluka Chemie AG Neu-Ulm, Switzerland. Ethylene glycol was from Acros Organics, Geel, Belgium.

Binding of soluble radiolabelled vWF to bacteria

vWF (50 μg) was labelled with Na^125I by a modified chloramine-T method (specificity, 2 × 10^5 cpm/μg) and used in a minor modified soluble binding test with final reaction volume of 500 μl in PBS [7]. During binding experiments, radiolabelled proteins were diluted to c. 0.1 μg (20,000 cpm) and incubated with bacterial suspension (10^9 cells). For saturation studies, bacteria (10^9 cells) were incubated with increasing amounts of ^125I-rvWF (maximally 25 μg) in 500 μl of PBS.

Heat treatment and proteolytic digestion

Bacteria (10^9 cells) were treated with Pronase E, proteinase K and trypsin as described previously [2]. For heat treatment, bacteria were heated at 100°C for 30 min and cooled in an ice bath. After treatment, bacteria were washed twice in PBS and incubated with 0.1 μg of ^125I-rvWF in 500 μl PBS.

Inhibition experiments

In the first group, bacteria (10^9 cells) were incubated with increasing amounts of unlabelled rvWF (maximally 50 μg) and 0.3 μg of ^125I-rvWF in 500 μl of PBS. In the second group, bacteria were incubated in the presence of 0.1 μg of ^125I-rvWF and 10 μg of competing proteins (fibrinogen, vitronectin and thrombospondin) or 0.1 M carbohydrates (N-acetylgalactosamine, d-galactose, d-glucose, d-fucose) in a 500-μl final reaction volume. In the third group, 0.1 μg of ^125I-rvWF was incubated with type 1 collagen 5 μg, heparin 250 μg and hyaluronic acid 250 μg for 30 min at 20°C in 400 μl of PBS. Then 100 μl (10^9 cells) of bacterial suspension were added for another hour. Alternatively, RGDS peptide (50 μg/p reaction tube) was mixed directly with ^125I-rvWF and bacteria.

Saturation study of rvWF to polystyrene (ELISA plate)

Two-fold dilutions of rvWF (0–100 μg/ml) in PBS were added in 100-μl volumes to the wells of an ELISA plate and held at 4°C overnight. The wells were saturated with bovine serum albumin (BSA) 3% in PBS and washed three times with Tween 20 0.01% in PBS (PBST), and the anti-vWF rabbit serum diluted 1 in 500 in PBST was added and incubated for 2 h at 20°C. The wells were washed and 100 μl of peroxidase-conjugated swine anti-rabbit immunoglobulins diluted 1 in 2000 in PBST were added. After incubation for 2 h and washing, the reaction was developed in the dark in 100 μl of a mixture comprising phenylenediamine 10 mg dissolved in 25 ml of citric acid buffer (pH 5.0) with 5 μl of H_2O_2 30%. The stopping solution was 1 M H_2SO_4. The absorbance values at 450 nm were measured in a spectrophotometer (Labsystem Multi-skan™ PLUS, Labsystems OY, Finland).
Bioluminescence assay for bacteria binding to immobilised rvWF

rvWF was immobilised on detachable ELISA plates (2 μg/well). Binding was quantified with a luminometer (LKB Wallac 1250 Luminometer, Turku, Finland) [17]. The values were expressed as percentage of retained adenosine triphosphate (ATP) from bound bacteria in relation to total added ATP produced by 100 μl of bacterial suspension (1 × 10⁷ cells). Wells coated only with BSA served as background. The values of these were subtracted from the values of other wells in the experiment before the percentages of binding were calculated.

SDS-PAGE, immunoblot assay and blocking tests

SDS-PAGE was performed under reducing conditions with a mini-Protean II cell (BioRad, Richmond, CA, USA). The bacterial surface proteins were extracted by 1 m LiCl with protease inhibitor (pH 5.0) at 37°C for 2 h. Crude extract (15 μg) was loaded into each well and separated in a homogeneous polyacrylamide 7.5% gel. The running and transfer conditions were as described previously [18, 19].

The membranes were saturated by overnight incubation with BSA 3% PBS containing Tween 20 0.1% at 4°C and then rinsed with PBST. The membranes were transferred to protein solutions (2 μg/ml in PBST) and held at 4°C for 16 h with gentle shaking. After three washes, primary antibodies to rvWF or thrombospondin diluted 1 in 500 in washing buffer (20 m M Tris buffer, pH 8.6, containing gelatin hydrolysate 0.5%, Tween 20 0.1%, 350 mM NaCl) were added and incubated for 2 h at 20°C [20]. The membranes were washed three times and incubated with peroxidase-conjugated swine anti-rabbit immunoglobulins diluted 1 in 2000 in washing buffer for another 2 h. After repeated washing, membrane-bound materials were detected by incubation in 50 mM sodium acetate buffer (pH 5) containing 3-amino-9-ethylcarbazole 0.04% and H₂O₂ 0.015%.

For blocking tests, the membranes with separated extract were incubated with rvWF 2 μg/ml mixed with thrombospondin 20 μg/ml or with thrombospondin 2 μg/ml, mixed with rvWF 20 μg/ml, at 4°C overnight. Binding structures were detected by anti-rvWF and anti-thrombospondin, respectively.

Statistical analyses

All data were given as mean and SEM. The two-tailed Mann-Whitney U test was used when appropriate; p < 0.05 was regarded as significant.

Results

Comparison of culture media

First, optimal culture conditions were determined for binding to rvWF. Two staphylococcal strains from different species and isolated from different kinds of biomaterial infections plus one reference strain of S. aureus were grown on or in blood agar, agar base, brain heart infusion (BHI) agar, Todd Hewitt (TH) broth, trypticase soy (TS) broth or Mueller-Hinton (M-H) broth. Bacteria grown on solid media bound rvWF to a greater extent. Growing Cowan I strain on both supplemented agar bases (blood agar and BHI agar) enhanced its binding level significantly and H2-W binding was promoted when bacteria were cultured on blood agar. SM 131 binding was not influenced by growth on different solid media (Fig. 1).

Binding of soluble radiolabelled rvWF to bacteria

All staphylococci selected for soluble binding experiments expressed binding of rvWF at percentages between 33% and 15% (Table 1). The binding extent of the S. aureus group was significantly greater than that of the CNS group (p < 0.001). Two Micrococcus spp. showed < 5% binding. The interaction between rvWF and bacteria reached a maximum after 10 min; inclusion of ethylene glycol 15% in the reaction solution did not affect binding. The S. epidermidis H2-W binding of rvWF showed a dose relationship that increased with increasing amounts of rvWF. However, even after adding rvWF, 50 μg/ml binding was not saturated (Fig. 2).

Heat treatment and proteolytic digestion

Cells of S. epidermidis strain H2-W and S. haemolyticus strain SM 131 were tested for susceptibility of rvWF binding to various proteinases and heat treatment. Proteinase treatment decreased the binding significantly. Heating increased the binding of H2-W to 110%, and reduced that of SM 131 to 80% (Table 2).

Inhibition experiments

Adding unlabelled rvWF at increasing concentrations up to 100 μg/ml inhibited the binding of strain H2-W to 125I-labelled rvWF (Fig. 3). Human vitronectin and fibrinogen did not influence the binding between strains Cowan I, SM 131 and H2-W and rvWF. Human thrombospondin decreased the relative binding by almost 75% (Fig. 4). None of the carbohydrates (N-acetylgalactosamine, α-galactose, β-glucose, β-fucose) blocked the binding. When rvWF was pre-incubated with heparin, binding by bacteria was reduced by > 50%, but addition of the same amount of hyaluronic acid did not influence binding. Pre-incubation with collagen type I enhanced the binding, particularly for S. aureus, which showed two-fold enhancement. The RGDS peptides did not have any inhibitory effect (Fig. 5).

Saturation study of rvWF to polystyrene (ELISA plate)

The adsorption of rvWF to the wells of ELISA plates was increased in a dose-dependent manner and reached saturation level between 12.5 and 25 μg/ml, which is
very close to the human plasma vWF concentration. A level of 20 µg/ml rvWF in each tube was chosen for coating.

Bioluminescence assay for bacterial binding to immobilised rvWF

Eight strains, including reference strains Cowan I, Wood 46 and B11653, were tested [12]. Five CNS strains bound immobilised rvWF to a significantly greater extent than the negative strains Wood 46 and B11653, and the highest binder was S. epidermidis H9-E (p < 0.01 compared to positive control Cowan I). Bacterial adhesion to the wells coated only with blocking agent (BSA 3% in PBS) was 5–35-fold lower than to rvWF-coated wells (Fig. 6).

SDS-PAGE, immunoblot assay and blocking tests

Crude extracts from strains Cowan I, SM 131 and H2-W were subjected to SDS-PAGE. rvWF binding molecules were identified in Cowan I at 140, 90 and 38 kDa, in SM 131 at 120 and 60 kDa and in H2-W at 120, 90, 60, 52 and 38 kDa by immunoblot. The band around 55 kDa was shown to be protein A, as this was the only band observed when rvWF was omitted, and
the membrane was incubated with antibodies only, data not shown. Binding structures of similar mol. wt were shown after incubation of extracts with thrombospondin, except that one more binding mass around 260 kDa was exhibited by strain SM 131. The intensity of these bands was reduced when these two proteins were incubated simultaneously and competed with each other (Fig. 7).

Discussion

The pathogens most frequently isolated from biomaterial-associated infections are CNS [21]. After implantation of foreign bodies, tissue proteins are adsorbed to the surface of the implant. Studies on the pathogenesis of device-associated infections must consider the characteristics of the medical device [22]. It has been well established that S. aureus can simultaneously express binding proteins for several host components, such as vitronectin, fibronectin, fibrinogen and heparan sulphate [7, 23–26]. Although binding of collagen type I, collagen type II, bone sialoglycoprotein, fibronectin, vitronectin and thrombospondin has been described, interactions between CNS and host factors are not fully understood [5, 26–31].

vWF is a glycoprotein, whose biological functions are primarily homeostatic and blood clot formation. Its mutations cause several variants of von Willebrand disease. As vWF has been detected on different biomaterials in contact with blood in vitro and in animal models [32, 33], it is reasonable to suppose that this protein could act as an adhesin for circulating bacteria. So far, binding of CNS to vWF have not been reported.

Because variation in the expression of cell wall proteins of S. aureus grown on solid and in liquid media has been reported, the growth conditions required for optimal expression of rvWF binding were studied [34]. Generally, bacteria grown on solid media bound rvWF to a greater extent than after growth in liquid media. Nutrient-poor conditions, which promote microbial adhesion to tissue or solid surfaces, did not enhance binding of all species [35]. In the present study 14 Staphylococcus strains, including reference strains, were tested for binding of soluble rvWF, and they all bound rvWF to varying extents. Two Micrococcus strains did not bind rvWF. The binding between S. epidermidis H2-W and rvWF was dose-related, but not saturated at concentrations up to 50 µg/ml. rvWF concentrations ≥ 100 µg/ml may be required to obtain
saturation, but these concentrations are difficult to evaluate because of the formation of protein–protein complexes yielding increased background values and because of multiple protein–bacteria interactions. Furthermore, as shown by immunoblot, there may be multiple binding sites on the bacterial surface. This could also explain why the display binding kinetics typical for a single receptor were not obtained. Binding

Fig. 3. **S. epidermidis** H2-W (1 × 10⁷ cells) was incubated with two-fold increasing concentration of unlabelled rvWF (from 0 to 100 µg/ml) mixed with labelled rvWF 0.6 µg/ml for 1 h at 20°C. Data are shown as mean values (n = 4) of triplicate samples tested twice. Insert: incubation with rvWF 0–0.2 µg with a different scale on the x-axis.

Fig. 4. Bacteria incubated with ¹²⁵I-rvWF 0.2 µg/ml alone (control; □) and with vitronectin (■), fibrinogen (□) or thrombospondin (□), 20 µg/ml each. The relative binding percentage was calculated (n = 6). Significant differences compared to control groups are indicated: **p < 0.01.
proteins of CNS strains are obviously distinct from those of S. aureus according to their molecular sizes. However, these different proteins probably contain similar amino-acid sequences, which may become exposed after proteolysis and participate in interaction. Binding could be inhibited by three different proteases, indicating that binding is mediated by proteinaceous structures in the bacterial cell wall. As rvWF contains a high proportion of N-glycans composed of mannose, galactose, glucose and N-acetyl glucosamine [14], these

Fig. 5. Bacteria incubated with $^{125}$I-rvWF 0.2 $\mu$g/ml alone (control; ) or after pre-incubation with collagen type-1 ( ), heparin ( ), hyaluronic acid ( ), or incubated with $^{125}$I-rvWF plus RGDS peptide ( ) in 500 $\mu$l of PBS. The reaction volume was 500 $\mu$l. The relative binding percentage was calculated ($n = 6$). Significant differences compared to control groups are indicated: * $p < 0.05$, ** $p < 0.01$.

Fig. 6. Adhesion of seven staphylococcal and one Micrococcus strain (c. $10^7$ cells added in each well) to rvWF-coated wells. Cowan I, Wood 46 and B11653 served as positive and negative controls, respectively. * $p < 0.05$ compared to other groups. ** $p < 0.01$ compared to Cowan I. Triplicate samples were made and repeated twice.
four carbohydrates were chosen for inhibition experiments. In spite of the fact that none of them inhibited binding, carbohydrate-mediated interaction could not be excluded, because the biological behaviour of saccharides may depend on their chain length [36]. It is well known that the surface hydrophobicity common to S. aureus as well as to strains of certain CNS species involves protease- and heat-sensitive surface structures [37]. In the present study, heat treatment decreased binding of SM131 only slightly. Ethylene glycol did not inhibit binding. This indicates that the activity could not be attributed predominantly to hydrophobic interaction.

The present study showed that the binding of soluble rvWF could be decreased by > 80% by pre-incubation with human thrombospondin. From the immunoblot results binding structures for these two glycoproteins of different staphylococcal species have similar mol. wts. S. aureus and CNS binding of thrombospondin have been well established [31]. The mechanism mediating binding between bacteria and these two proteins might be very similar. In this case, N-terminal sequences of these binding structures should be investigated.

vWF possesses distinct binding domains for several human macromolecules, such as for collagen, heparin and platelet (GPIb and GPIIb-IIIa). Human collagen type I and heparin were chosen to block the domains on the rvWF molecule, respectively. Collagen type I enhanced bacterial binding of rvWF. As staphylococcal binding of collagen type I has been reported previously [2], it is speculated that the collagen binding domain(s) are not involved in the binding. The bound collagen on these domains could serve as extra binding sites for bacteria or act as a ‘bridge’ between rvWF molecules to form protein–protein complexes. Heparin decreased binding significantly, but hyaluronic acid did not. The inhibition of staphylococcal binding of rvWF only by heparin may indicate that heparin blocked these binding domains rather than acting only through its high negative charge, possibly by its sulphate groups. Another explanation would be that the binding molecules in the bacterial cell not only recognised rvWF but also heparin. It is known that S. aureus can express binding of heparin and that CNS strains do not bind soluble heparin or bind it to a very low extent [23]. As the binding of S. aureus and CNS strains was decreased to a similar level in the experiment, the second possibility is less likely. The RGDS-dependent cell attachment domain of rvWF was apparently not involved in binding, because RGDS peptides did not block the interaction.

Saturation adsorption kinetics were found when rvWF was coated on a polystyrene surface. All CNS strains could attach to immobilised rvWF to a greater extent. Strain H9-E presented a two-fold higher binding level than that of S. aureus Cowan I, suggesting that the protein in its surface-bound conformation expresses cryptic binding sites for CNS.

Domain A1 of adsorbed rvWF on subendothelial stroma or the surface of prosthetic devices contains binding sites for several macromolecules, including platelet GPIb and heparin [8]. It is reasonable to speculate that domain A1 blocked by attached bacteria could lose its normal biological functions for binding of non-activated platelets. On the other hand, activated platelets do not seem to be affected by bacterial adhesion, because bacteria do not bind to their binding domain (RGDS).

In conclusion, rvWF in solid and fluid phase bound to coagulase-negative staphylococci and this interaction was predominantly protein-mediated. Multiple binding molecules recognised rvWF, and these components also bound human thrombospondin. Regions for bacterial attachment on the rvWF molecule appear to be close to
the heparin binding domain, but distinct from the eukaryote cell-binding domain. The property of rvWF as a promoter of bacteria adhesion could bring about the possibility of its physiological form, vWF, acting as a mediator between CNS and intravascular biomaterial surfaces.

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References


