BACTERIAL PATHOGENICITY

Role of *Staphylococcus aureus* surface adhesins in orthopaedic device infections: are results model-dependent?

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Bacterial colonisation of prosthetic material can lead to clinical infection or implant failure, or both, often requiring removal of the device. Adherence of *Staphylococcus aureus* to bioprosthetic materials is mediated by adhesins belonging to the MSCRAMM (microbial surface components recognising adhesive matrix molecules) family of microbial cell surface proteins. The objective of this study was to compare the virulence of a mutant strain of *S. aureus* Newman that possesses all three fibrinogen-, fibronectin- and collagen-binding MSCRAMMs (MSCRAMM-positive strain) with that of a mutant strain that lacks all three types of MSCRAMMs (MSCRAMM-negative strain) in a rabbit model of orthopaedic device-related infection. After a hole was drilled into the knee joint of each animal, a group of 10 rabbits was inoculated with the MSCRAMM-positive strain and another group of 10 rabbits received the MSCRAMM-negative strain. A stainless steel screw was then placed into the drilled hole. Two weeks later, the rabbits were killed and serum samples, bone tissue and implants were harvested for bacteriological and histopathological evaluation. No significant difference in infection rates was demonstrated between the two groups. The ability to delineate the role of *S. aureus* surface adhesins in causing orthopaedic device-related infection could be model-dependent.

Introduction

Despite strict adherence to sterile procedures and aggressive use of antimicrobial prophylaxis, infection remains a major complication of orthopaedic devices. Infection rates of joint prostheses range from as low as 0.06% in patients with primary hip replacements to as high as 11.8% in patients who have had previous hip surgery [1, 2]. Open fractures comprise an even greater problem and recent studies have shown that the incidence of infection following stabilisation of such fractures can be as high as 13.9% [3]. Coagulase-negative and coagulase-positive staphylococci are responsible for the majority of cases of infection associated with orthopaedic implants. For instance, *Staphylococcus aureus* was reported to be the most frequently isolated organism in infections related to total hip replacement [4] and has been reported to cause 25–50% of infections following open fractures [5]. The predominant role of *S. aureus* in causing osteomyelitis is well founded, being the sole causative organism in >55% of cases of osteomyelitis [6].

*S. aureus* can colonise a wide variety of host tissues and medical devices and cause various types of infections such as endocarditis, bacteraemia, osteomyelitis and septic arthritis. Adherence of *S. aureus* to host tissues and biomaterials involves the microbial surface components recognising adhesive matrix molecules (MSCRAMM) family of microbial cell surface proteins that specifically recognise host extracellular matrix and plasma proteins [7]. The adherence of *S. aureus* to host tissues and plasma proteins is thought to be a critical factor for the development of infection at the site of an implant. After implantation, biomaterials rapidly become coated with host plasma proteins, predominantly fibrinogen (Fbg) and fibronectin (Fn). Fbg, a 340-kDa glycoprotein composed of six
polypeptide units (α2β2γ2), is found in high concentrations in plasma and plays important roles in blood coagulation and wound healing. Fbg quickly coats the external surface of most biomaterials that are implanted into the body. Deposition on foreign bodies and at sites of trauma allows fibrin/fibrinogen to potentially serve as a substrate for microbial adherence [8]. The adherence of *S. aureus* to Fbg-coated biomaterials is primarily mediated by aMSCRAM called clumping factor (ClfA) [9]. Similarly, Fn, a glycoprotein found on the surface of most cells, in the extracellular matrix of many tissues as well as in body fluids such as plasma, has been shown to influence the adherence of *S. aureus* to foreign materials in a strain-specific pattern [8]. Attachment of *S. aureus* strain 8325-4 to Fn is mediated by cell surface Fn-binding MSCRAMMs FnbpA and FnbpB encoded by the genes fnbA and fnbB, respectively [10]. *S. aureus* also expresses a collagen-binding MSCRAMM that is both necessary and sufficient for attachment to collagen-containing substrata such as cartilage [11]. The gene cna that encodes the *S. aureus* collagen-binding MSCRAMM has been cloned and sequenced [12, 13].

The purpose of this study was to determine if the *S. aureus* expression of fibrinogen-, fibronectin- and collagen-binding MSCRAMMs enhances the ability of the organism to cause infection in a rabbit model of orthopaedic device-related infection. This research was done with the understanding that if these MSCRAMMs can be proved to be virulence determinants for orthopaedic device-related infection, such findings will then be used to launch future studies to evaluate the efficacy of various preventive measures, including the administration of antibodies directed against these extracellular matrix molecules.

**Materials and methods**

*S. aureus* strain Newman expresses the Fbg-binding MSCRAMM (ClfA) and the Fn-binding MSCRAMMs (FnbpA and FnbpB), but does not express the collagen-binding MSCRAMM (CNA). To construct a mutant strain that expressed all three types of MSCRAMMs, the cna gene was introduced into the chromosome of strain Newman with a single copy integrating vector [14, 15] creating strain Newman::pCL574 (designated as the MSCRAMM-positive strain which is FnbpA+, FnbpB+, ClfA+ and CNA+). A site-specific mutant of *S. aureus* strain Newman that does not express Fn-binding MSCRAMMs was created by replacing the chromosomal copies of fnbA and fnbB gene loci with the mutated alleles from *S. aureus* strain DU5883 (8325-4 fnbA fnbB) [16] via phage transduction. The transductants were selected on tetracycline agar and screened for the fnbB:Emr mutation by PCR as described previously [16]. To construct a version of strain Newman that was also deficient in the expression of the Fbg-binding MSCRAMM, the fnbA:Trc fnbB:Emr double mutation was co-transduced from strain DU5883 into a ClfA- version of strain Newman (DU5852) [9] to form strain DU5886. The transductants were identified as described earlier. The resulting strain DU5886 (designated as the MSCRAMM-negative strain which is FnbpA-, FnbpB-, ClfA- and CNA-) failed to clump in the presence of a fibrinogen 1% solution and did not bind 125I-labelled collagen type II and fibronectin (data not shown).

Twenty adult female, New Zealand White rabbits (weight 3.5–4.0 kg) were anaesthetised with ketamine 0.375 ml/kg injected intramuscularly in a solution containing ketamine 42.8 mg/ml, xylazine 8.6 mg/ml and acepromazine 1.4 mg/ml. To better simulate perioperative antibiotic prophylaxis in man, all rabbits received one intramuscular dose of cefazolin 80 mg/kg 30 min before surgery. This relatively high prophylactic antibiotic dose (about four times higher than that used in man) was necessary because of the shorter half-life (30–40 min) of cefazolin in rabbits. Both the MSCRAMM-positive and MSCRAMM-negative strains were sensitive to cefazolin by the Kirby-Bauer disk diffusion method, and the minimum inhibitory concentration (MIC) of cefazolin for each of these two bacterial strains was determined by standard broth macrodilution assay to be 1 mg/L.

The right hind leg of each rabbit was shaved, prepared and draped in a sterile manner; 5 ml of lidocaine 1% was injected locally. A 2-cm incision was made over the medial knee joint by aseptic techniques. The patella was dislocated laterally, and the knee joint and intercondylar notch were exposed. A sterile hand drill loaded with a 3.2-mm drill bit was then used to produce a hole through the intercondylar notch into the medullary canal of the femur. After controlling bleeding from the medullary canal, a sterile pipette tip containing 0.1 ml of bacterial suspension of *S. aureus* (10^6 cfu/ml) that had been washed and resuspended in phosphate-buffered saline (PBS) was introduced into the drill hole; pilot experiments demonstrated previously that 10^4 cfu of the MSCRAMM-positive strain of *S. aureus* was the lowest bacterial inoculum that would result in infection in this animal model. The first group of 10 rabbits (group A) was inoculated with the MSCRAMM-positive strain of *S. aureus*. The second group of 10 rabbits (group B) was inoculated with the MSCRAMM-negative strain of *S. aureus*. A 3.5 × 18-mm stainless steel screw was inserted into the drill hole and the wound was closed primarily.

Rabbits were monitored daily, particularly with regard to temperature, signs of sepsis and body weight. After an incubation period of 2 weeks, the animals were killed by intracardiac injection of phenobarbital. The following specimens were collected in a sterile fashion and cultured by inoculating on to chocolate blood agar plates (BBL media; Becton Dickinson Microbiology
Systems, Cockeysville, MD, USA): 10-μl blood samples from rabbit heart; swab of synovial fluid from the inoculated knee; swab of implant track; stainless steel screw implant (by roll-plate culture technique); 10-μl volumes of original sample of biofilm from around implant and of serial dilutions (after rolling the implant over an agar plate, the implant was weighed, then immersed in trypsin 0.5% solution for 30 min to detach the biofilm, then weighed dry again; the difference between the two recorded weights of implant reflected the weight of detached biofilm); swab of bone-implant interface; and 10-μl volumes of original sample of homogenised bone tissue and of serial dilutions (femur bone was split longitudinally into two halves; one half was submitted for histopathological examination and the second half was homogenised in PBS at 4°C).

Bone specimens for histopathological examination were fixed in formalin 10% for at least 2 days. After decalcification for 2–3 days, sections parallel to the long axis of the bone were processed in a standard fashion by dehydration into paraffin until the sections were permeated thoroughly. Bone specimens embedded in paraffin blocks were cut into 5-μm thick sections, mounted on a glass slide and stained with haematoxylin-eosin. Each section was examined by the same pathologist and evaluated for the presence or absence of acute inflammation, chronic inflammation, new bone formation, type of periosteal reaction, necrosis, abscess, dense bone formation, intra-osseous fibrosis, etc. The findings were graded semiquantitatively from 1+ to 3+. Because evaluation of decalcified tissue by light microscopy may be suboptimal and various cytological features of cells present in the sections may be obscured, touch preparations of the cut bone surfaces were also made before specimens were placed in formalin.

Isolation of the inoculated strain of \textit{S. aureus} from cultured specimens was considered to indicate the presence of infection, irrespective of the concentration of isolated bacteria. Rates of infection were statistically analysed by Fisher’s exact test; the unpaired Student’s \textit{t}-test was used to compare the results of quantitative bacterial cultures.

**Results**

One animal in group B died 4 days after the operation, presumably due to sepsis, with a temperature > 42°C and was excluded from statistical analysis. In each infected rabbit, all positive bacterial cultures yielded the same \textit{S. aureus} strain that had been inoculated originally. As indicated in Table 1, infection rates of blood, synovial fluid, implant track, implant (by roll-plate technique), biofilm around implant, bone-implant interface and bone were not significantly different in the two groups of rabbits. Moreover, among rabbits with infected bone, the mean bacterial concentrations were identical (4.6 × 10^5 cfu/g of bone tissue) in the two groups of rabbits. The mean bacterial concentrations in infected biofilm samples were also not significantly different in rabbits that received the MSCRAMM-positive strain compared to rabbits that received the MSCRAMM-negative strain (5.4 × 10^3 cfu/g versus 1.6 × 10^4 cfu/g, respectively).

Histopathological examination of every bone specimen that had yielded a positive bacterial culture revealed changes consistent with acute inflammation, sequestrum formation, new bone formation, periosteal reaction, necrosis and fibrosis; however, histopathological examination did not reveal bacteria in any bone specimen. Although acute inflammatory changes were noted in every specimen (whether infected or non-infected), changes were generally more prominent in culture-positive specimens (grade 2+ to 3+) than in culture-negative specimens (grade 1+ to 2+). A similar trend was noted with regard to the intensity of periosteal reaction and dense bone formation in culture-positive versus culture-negative specimens. However, necrosis and abscesses were observed only in culture-positive bone samples. There were no consistent differences in histopathological findings in rabbits that received the MSCRAMM-positive strain compared to rabbits that received the MSCRAMM-negative strain.

**Discussion**

\textit{S. aureus}, one of the most common causes of orthopaedic device-related infection, was used in this animal model because it has been shown to bind to plasma-conditioned foreign biomaterials and extracellular matrix components. With other animal models of infection, MSCRAMM isogenic mutants of \textit{S. aureus} have been reported to be significantly less virulent than their MSCRAMM-positive counterparts. For example, a ClfA− strain of \textit{S. aureus} was significantly less virulent compared to its parental strain in an animal model of experimental endocarditis [17]. In a recent report, a strain of \textit{S. aureus} that was defective in the expression of a ClfA exhibited an 80% decrease in the

<table>
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<tr>
<th>Specimen</th>
<th>Number of positive cultures/total (%)</th>
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<tbody>
<tr>
<td></td>
<td>Group A</td>
</tr>
<tr>
<td>Blood</td>
<td>0/10 (0)</td>
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<tr>
<td>Synovial fluid</td>
<td>2/10 (20)</td>
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<tr>
<td>Implant track</td>
<td>6/10 (60)</td>
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<tr>
<td>Implant</td>
<td>5/10 (50)</td>
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<td>Biofilm around implant</td>
<td>3/10 (30)</td>
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<tr>
<td>Implant-bone interface</td>
<td>3/10 (30)</td>
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<tr>
<td>Bone</td>
<td>5/10 (50)</td>
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Differences between groups A and B were not statistically signi-
ability to attach to canine arteriovenous shunts [18]. A mutant strain of *S. aureus* that was defective in the expression of the fnbA and fnbB genes had significant impairment in its ability to adhere to coveslips removed from tissue cages implanted subcutaneously in guinea-pigs [15]. The role of extracellular matrix molecules has also been proved in an animal model of orthopaedic infection that was not device-related [15]. The expression of an intact cna gene by *S. aureus* organisms was found in that study to be a virulence determinant for haematogenously acquired septic arthritis in mice; >70% of mice inoculated with the CNA+ strains developed clinical signs of arthritis, whereas <27% of animals inoculated with the CNA− strains showed symptoms of disease [15]. Furthermore, an analysis of *S. aureus* isolates from patients with septic arthritis or osteomyelitis demonstrated that almost all strains possessed a collagen-binding adhesin [11]. In contrast, only one-third of *S. aureus* isolates from patients with soft tissue infection expressed the collagen adhesin [11].

The results of this animal study did not demonstrate a significant difference between strain Newman::pG7574 (MSCRAMM-positive strain) and strain DU5886 (MSCRAMM-negative strain) in their ability to cause an orthopaedic-device related infection. The rates of infection and the intensity of infection (as reflected by the mean bacterial concentrations in infected bone tissues) were similar in the two groups of rabbits. It is tempting to speculate why a difference in the virulence between the two strains of *S. aureus* was not observed in this animal model of orthopaedic device-related infection but could be delineated in the previous animal study of haematogenously acquired septic arthritis [15]. It is possible that, in the present study, inoculation of bacteria directly into the knee joint effectively masked any differences between the strains in their ability to colonise the implant, bone and surrounding tissues. It is also important to note that of the animals surviving the 2-week follow-up period, none had positive blood cultures, indicating that this particular animal model of infection did not result in systemic sepsis; the only exception may have been a single rabbit in the group that received the MSCRAMM-negative strain which died apparently from sepsis 4 days after bacterial inoculation. Perhaps insertion of the implant followed by a haematogenous inoculation of the bacteria would have delineated differences in the adherence of the two bacterial strains; however, the majority of clinical cases of orthopaedic device-related infection are thought to be caused by organisms that are directly inoculated into the implant site some time during the perioperative period rather than haematogenously acquired.

The findings of this study serve to alert investigators that the results of virulence studies of MSCRAMMs in *S. aureus*-related orthopaedic device infections may be model-dependent. This suggestion is supported by a recent report that demonstrated opposing results when the infectivity of two *S. aureus* strains that had different virulence factors was compared in a rabbit model of acute osteomyelitis versus a murine model of peritonitis [19]. Further studies, with other models, will be necessary to fully explore the biological role of MSCRAMMs as determinants of virulence in *S. aureus*-related orthopaedic device infections. Only then can the ultimate goal of inhibiting bacterial adherence to these extracellular matrix molecules covering medical devices be assessed reliably for the prevention of orthopaedic device-related infections.

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References