Biomaterials 29 (2008) 4684–4690

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

The inhibition of *Staphylococcus epidermidis* biofilm formation by vancomycin-modified titanium alloy and implications for the treatment of periprosthetic infection

Valentin Antoci, Jr.[a], Christopher S. Adams[a], Javad Parvizi[a,b], Helen M. Davidson[a], Russell J. Composto[c,d], Theresa A. Freeman[a], Eric Wickstrom[e], Paul Ducheyne[c,f], Donald Jungkind[g], Irving M. Shapiro[a,c,e], Noreen J. Hickok[a,e,*]

[a] *Department of Orthopaedic Surgery, Thomas Jefferson University, Philadelphia, PA, USA*
[b] *The Rothman Institute, Philadelphia, PA, USA*
[c] *Center for Bioactive Materials and Tissue Engineering, School of Engineering and Applied Science, University of Pennsylvania, Philadelphia, PA, USA*
[d] *Department of Materials Science & Engineering, University of Pennsylvania, Philadelphia, PA, USA*
[e] *Department of Biochemistry and Molecular Biology, Thomas Jefferson University, USA*
[f] *Department of Bioengineering, University of Pennsylvania, Philadelphia, PA, USA*
[g] *Department Pathology, Anatomy, and Cell Biology Thomas Jefferson University, Philadelphia, PA, USA*

ARTICLE INFO

Article history:
Received 29 April 2008
Accepted 4 August 2008
Available online 23 September 2008

Keywords:
Antibacterial
Bacterial adhesion
Biofilm
Metal surface treatment
Bacteria
Surface modification
Titanium alloy

ABSTRACT

Peri-prosthetic infections are notoriously difficult to treat as the biomaterial implant is ideal for bacterial adhesion and biofilm formation, resulting in decreased antibiotic sensitivity. Previously, we reported that vancomycin covalently attached to a Ti alloy surface (Vanc-Ti) could prevent bacterial colonization. Herein we examine the effect of this Vanc-Ti surface on *Staphylococci epidermidis*, a Gram-positive organism prevalent in orthopaedic infections. By direct colony counting and fluorescent visualization of live bacteria, *S. epidermidis* colonization was significantly inhibited on Vanc-Ti implants. In contrast, the gram-negative organism *Escherichia coli* readily colonized the Vanc-Ti rod, suggesting retention of antibiotic specificity. By histochemical and SEM analysis, Vanc-Ti prevented *S. epidermidis* biofilm formation, even in the presence of serum. Furthermore, when challenged multiple times with *S. epidermidis*, Vanc-Ti rods resisted bacterial colonization. Finally, when *S. epidermidis* was continuously cultured in the presence of Vanc-Ti, the bacteria maintained a Vanc sensitivity equivalent to the parent strain. These findings indicate that antibiotic derivatization of implants can result in a surface that can resist bacterial colonization. This technology holds great promise for the prevention and treatment of periprosthetic infections.

1. Introduction

Implant surfaces serve as ideal substrates for bacterial colonization. This colonization often results in periprosthetic infection (PPI), a condition that leads to destruction of local tissues, patient disability and morbidity, and on occasion, death [1]. The implant surface accumulates serum proteins which promote bacterial adherence and colonization [2], and serves as a critical first step in the development of PPI. The adherent bacteria synthesize a complex glycocalyx that enmeshes the bacteria and provides escape for them from immune surveillance or antibiotic treatment [3–7]. Currently, PPI treatment requires removal of the contaminated implant coupled with extensive bone debridement, excision of infected tissues and bone, and prolonged antimicrobial treatment [8].

Various antimicrobial materials have been developed to counter this implant-associated infection. The gold standard for targeted local delivery of antimicrobial agents is antibiotic-impregnated bone cement (polymethylmethacrylate – PMMA); other antibiotic-impregnated biodegradable carriers such as sol–gel coatings [9,10] and hydrogels [11,12] are under development. The use of antibiotic loaded PMMA allows delivery of high concentrations of antibiotics to the affected area without causing systemic toxicity. There are, however, numerous problems with the current local antibiotic delivery systems. First, most local antibiotic delivery systems are, by their nature, targeted to initiate treatment upon implantation,

* Corresponding author. Department of Orthopaedic Surgery, Thomas Jefferson University, 1015 Walnut Street, Suite 501, Philadelphia, PA 19107, USA. Tel.: +1 215 955 6979; fax: +1 215 955 9159.
E-mail address: noreen.hickok@jefferson.edu (N.J. Hickok).

0142-9612/$ – see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biomaterials.2008.08.016

with levels of eluted drug remaining high for a well-defined period. Subsequently, the elution of antibiotics falls below the minimal inhibitory concentration for a longer period, a situation that could result in emergence of resistant organisms. Second, the drug delivery is ineffective in penetrating to the bacteria embedded within the biofilm on the implant surface. Finally, addition of many antibiotics to PMMA is not possible as the exothermic reaction caused by curing of the cement results in heat inactivation of the drug [13,14].

Attachment of bacteria to the implant surface appears to be the initial and the most critical step in development of PPI and hence, we and other investigators have invested extensive efforts to prevent bacterial adherence. We have previously reported that covalently bonding antibiotics to a metal implant surface can inhibit bacterial attachment. This antibiotic-derivatized surface is stable in the face of *Staphylococcus aureus* (*S. aureus*) challenges and prevents its colonization [15]. In this report, we examine the effect of this surface on a strain of *Staphylococcus epidermidis* (*S. epidermidis*, ATCC™ 155®), an organism that is prevalent in orthopaedic infections and notorious for forming biofilms. Furthermore, we evaluate the ability of this surface to prevent bacterial colonization in the presence of serum proteins. Results of the study indicate that derivatization of implant surfaces with antibiotics may provide a practical approach to inhibiting bacterial colonization of implants and thereby minimizing PPI.

2. Materials and methods

2.1. Experimental design

Colonization of the surface of vancomycin-modified Ti alloy (Ti90Al6V4) rods (Vanc-Ti) by *S. epidermidis* was evaluated. We chose Ti90Al6V4 for these studies as it is commonly used for manufacturing orthopedic implants. Surface colonization was measured by determining the numbers of *S. epidermidis* colony forming units (cfu) adherent to the rods and by treatment of the surface with a vital stain. In parallel, *S. epidermidis* colonization of the surface was visualized using scanning electron microscopy (SEM), currently the gold standard for biofilm detection. The specificity of inhibition was evaluated using the gram negative organism *E. coli*, an organism that is not sensitive to this antibiotic. Finally, we determined if there was emergence of resistant strains of *S. epidermidis* on the Vanc-Ti rods following multiple bacterial challenges.

2.2. Antibiotic modification of Ti alloy

One mm diameter Ti90Al6V4 wire (Ti alloy, Goodfellow, Cambridge, UK) was passivated with H_2O_2/H_2SO_4 and aminopropylated under argon using 5% (v/v) aminopropyl-triethoxysilane in anhydrous toluene. It was coupled sequentially with two Fmoc-[2-(2-amino-ethoxy)-ethoxy]-acetic acid (AEEA) linkers and vancomycin in the presence of O-(7-azabenzo-triazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU); between each addition, deprotection was achieved with 20% piperidine in N,N-dimethylformamide [15–17].

2.3. Evaluation of surface colonization

Control and Vanc-Ti rods (5–10 mm × 1 mm) were weighed, sterilized with 70% EtOH for 15 min, and washed twice with BBL™ Trypticase™ Soy Broth (TSB, BD Bioscience). *S. epidermidis* ATCC™ 155® or *E. coli* DH5α® was cultured in TSB, 250 rpm, 37 °C, 12–16 h (overnight culture) and diluted to 1×10^4 cfu/ml using a 0.5 McFarland standard, a turbidity standard which is equivalent to 10^7–10^8 cfu of *S. aureus* (PML Microbiologicals, Wilsonville, OR). To evaluate surface colonization, 0.5 ml of the diluted culture was incubated with rods for 2, 5, 8, 12, or 30 h at 37 °C under static conditions. At each time period, 5 control and 5 Vanc-Ti rods were removed. Each rod was washed 7 times with phosphate-buffered saline (PBS), to remove non-adherent bacteria. Two rods were used to determine surface bacterial adhesion/viability and 3 were used for measurement of numbers of adherent bacteria. Additionally, 3 control and 3 Vanc-Ti rods per time point were processed for SEM.

2.4. Effect of serum on microbiocidal activity

Control and Vanc-Ti rods were incubated with complete fetal bovine serum (FBS, Atlanta Biochemicals) or dH_2O statically at 37 °C for 24–48 h. Rods were removed and either fixed with 4% paraformaldehyde for 5 min and immunostained for fibronectin, or incubated with 1×10^4 cfu/ml *S. epidermidis* for 24 h and evaluated as above.

2.5. Determination of biofilm formation

Biofilms were stained with crystal violet using a modification of the method of O'Toole et al. [18]. Specifically, 0.5 ml ($C_i = 10^4$ cfu/ml) of *S. epidermidis* cultures were statically incubated in 24 well plates containing control or Vanc-Ti rods for 2, 5, or 8 h at 37 °C. The medium containing non-adherent bacteria was carefully removed and Vanc-Ti and control rods moved to fresh wells. These rods were washed 6X with PBS and then stained, in a new well, with 1 ml of PBS containing 100 μl of 1% (w:v) crystal violet (Sigma-Aldrich, St. Louis, MO) and incubated for 15 min at room temperature. The rods in the wells were washed 3 times with PBS and crystal violet then solubilized by addition of 2 ml of 95% EtOH, with rocking for 15 min; absorbance was measured ($\lambda = 570$ nm) using a SPECTRAFluorPlus fluorimeter (Tecan, Research Triangle Park, NC).

2.6. Rod activity following repeated bacterial challenges

Control or Vanc-Ti rods that had been incubated with *S. epidermidis* in TSB for 24 h were cleaned by washing with 1% Triton X-100 and 10% sodium lauryl sulfate (Fisher Scientific) in dH_2O for 24 h. They were then washed 5 times with PBS, sterilized with 70% EtOH for 15 min, and washed twice with TSB. Cleaned rods were incubated with 1×10^4 cfu/ml *S. epidermidis* in TSB at 37 °C for 24 h. Following washing 5 times with PBS, the rods were assayed for bacterial adhesion/viability (first re-challenge). Each re-challenge involved an additional round of washing and culturing with *S. epidermidis* as described above.

2.7. Evaluation of development of resistance

S. epidermidis was cultured ($C_i = 10^4$ cfu/ml) in TSB and incubated with the Vanc-Ti rods for up to 8 weeks, with TSB replaced every 3–4 days. Weekly, the rods were removed from the medium, sonicated in TSB to suspend adherent bacteria, and 200 μl plated on agar plates. These colonies were assessed by the Thomas Jefferson University Clinical Microbiology Labs for resistance using growth on vancomycin plates (6 μg/ml) as well as the Kirby-Bauer disk diffusion assay.

2.8. Quantitative measure of adherent bacteria

Adherent bacteria were detached by sonication for 10 min in 1 ml TSB, followed by vortexing for 2 min, using a modification of the method of Barth et al. [19]. Total numbers of bacteria were determined by serial dilutions on TSB agar plates; the plates were incubated at 37 °C for 24 h, and colonies counted. For detection of slow growing colonies, those plates exhibiting no growth after 24 h were incubated for an additional 24 h. Numbers of released bacteria were calculated, based on dilution, and expressed as a function of rod weight.

2.9. Fluorescent staining of adherent bacteria

To remove non-adherent bacteria, rods were washed 7 times with PBS. These were stained using the Live/Dead® BacLightTM Viability Kit (Invitrogen) for 15 min to fluorescently label viable bacteria, followed by three PBS washes to remove nonspecific stain. Fluorescent, adherent bacteria were visualized by confocal laser microscopy (Olympus Fluoview 300).

2.10. Immunohistological detection of vancomycin and fibronectin

Rods were washed twice with PBS and incubated with a primary antibody against vancomycin (mouse anti-vancomycin IgG, 1:500, US Biologicals) in PBS 4 °C, 2 h. For visualizing fibronectin, after incubation in FBS, rods were fixed with 4% paraformaldehyde, incubated with rabbit anti-bovine fibronectin IgG (1:500, Invitrogen) in PBS, 4 °C, 2 h. They were then washed 3 times with PBS, incubated in PBS for 30 min, and then treated with AlexaFluor 488-coupled donkey anti-rabbit or anti-mouse IgG (1:500, Invitrogen) in PBS, 1 h, followed by 5 PBS washes and incubation in PBS for 30 min. Stain was visualized by confocal laser microscopy (Olympus Fluoview 300).

2.11. Scanning electron microscopy

Vanc-Ti rods were incubated with *S. epidermidis* for 24 h in TSB. Samples were washed extensively up to 6 times in PBS and fixed with 2.5% glutaraldehyde in 0.1 mol/L piperazine-1,4-bis(2-ethanesulfonic acid) buffer (pH 7.4) for 5 min. Following fixation, the samples were stained with 1% OsO_4 in 0.1 mol/L piperazine-1,4-bis(2-ethanesulfonic acid) buffer (pH 6.8) for 1 h and then re-stained with 2% uranyl acetate for 1 h. The rods were dehydrated in ethanol and sputter-coated with gold. Surfaces were visualized using an FEI Strata™ DB235 focused ion beam SEM (FEI Co, Hillsboro, OR).

2.12. Statistics

Experiments were performed a minimum of 3 independent times using at least 3 randomly chosen samples for each repeat. To establish significance, a one-tailed Student's *t* test was used ($^*p < 0.05$). The results were tested by the Kolmogorov–Smirnov normality tests.