Biomaterials 30 (2009) 3158–3166

Contents lists available at ScienceDirect

Biomaterials

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

The effect of photodynamic treatment combined with antibiotic action or host defence mechanisms on *Staphylococcus aureus* biofilms

Antonella Di Poto [a], Maria S. Sbarra [a], Giulio Provenza [a], Livia Visai [a,b], Pietro Speziale [a,*]

[a] University of Pavia, Department of Biochemistry, Viale Taramelli 3/b, 27100 Pavia, Italy
[b] University of Pavia, Center for Tissue Engineering, Via Ferrata 1, 27100 Pavia, Italy

ARTICLE INFO

Article history:
Received 19 December 2008
Accepted 23 February 2009
Available online 28 March 2009

Keywords:
Staphylococcus
Biofilm
Photodynamic treatment
Vancomycin
Phagocytosis

ABSTRACT

Staphylococcus aureus is one of the most important etiological agents of infections associated with medical devices. This is in part due to the ability of the organism to form biofilm, which provides a microenvironment that protects from attack by the host's immune system and by antibiotics. In this study we examined the structure of polysaccharide intercellular adhesin (PIA)-dependent or protein-based *S. aureus* biofilms. We defined new strategies aimed at treatment of mature established biofilms using photodynamic treatment (PDT) combined with chemotherapy or phagocytosis. Significant inactivation of bacteria was observed when structurally distinct biofilms were exposed to the cationic porphyrin, tetra-substituted *N*-methyl-pyridyl-porphine (TMP), and simultaneously to visible light. Moreover, PDT-treated biofilms exposed to vancomycin or subjected to the phagocytic action of whole blood resulted in their almost complete eradication. The drastic reduction in staphylococcal survival and the disruption of biofilms were confirmed by confocal laser scanning microscopy and scanning electron microscopy. The results suggest that PDT combined with vancomycin and the host defences may be a useful approach for the inactivation of staphylococcal biofilms adhering to medical implant surfaces.

1. Introduction

Among staphylococci, *Staphylococcus aureus* is regarded as the most virulent species due to its large variety of virulence factors [1]. A hallmark of chronic *S. aureus* infections is the ability of bacteria to grow as a biofilm, a sessile community of cells that is embedded in an extracellular polymeric matrix. Biofilm forms when bacteria attach to a solid surface, proliferate and develop into multicellular layers [2]. The best characterized staphylococcal biofilms involve the polysaccharide intercellular adhesin (PIA), a linear homoglycan composed of at least 130 residues of β-1,6-linked *N*-acetylglucosamine, 15–20% of which are deacetylated and therefore positively charged. PIA is also a crucial element in biofilm formation for many strains of *S. epidermidis*, an important cause of foreign body infections [3]. PIA is produced by genes of the *icaRADBC* locus, an operon of four biosynthetic genes (*icaADBC*) [4] and *icaR* [5], encoding a divergently transcribed negative regulator protein.

Recently, it has become apparent that *ica*-independent mechanisms of biofilm formation occur in *S. aureus* and *S. epidermidis*. The biofilm-associated protein (Bap) is essential for biofilm development of *S. aureus* strains isolated from bovine mastitis strains [6]. The *S. aureus* surface protein SasG (surface-associated *Staphylococcus* protein G) also promotes the development of PIA-independent biofilm [7]. A study performed on MRSA strains has identified a novel biofilm phenotype promoted by fibronectin-binding proteins FnBPA and FnBPB [8]. Extracellular DNA contributes structurally to *S. aureus* biofilms [9]. Because of its polyanionic nature, it is plausible that DNA binds together other molecules such as PIA in the biofilm matrix.

The ability to form biofilm is a crucial factor in persistent infections caused by staphylococci. Once established, these communities constitute a protective niche that promotes growth and survival of bacteria in a hostile environment and are difficult to treat due to their resistance to antibiotics, possibly due the antibiotic failure to permeate the biofilm [10]. A similar resistance to innate and adaptive host immune defences has been described [11–13]. Along this line, an immune therapeutic approach based on the efficient internalization and killing of bacteria by macrophages and polymorphonuclear leucocytes has been used for treating infections resulting from antibiotic resistant microorganisms, but the level of phagocytic killing of staphylococci in the biofilm matrix resulted significantly lower compared to the opsonophagocytosis of planktonic cells [14].

* Corresponding author. Department of Biochemistry, Faculty of Medicine and Surgery, University of Pavia, Viale Taramelli 3/b, 27100 Pavia, Italy. Tel.: +39 0382 987787; fax: +39 0382 423108.
E-mail address: pspeziale@unipv.it (P. Speziale).

0142-9612/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biomaterials.2009.02.038

Modern medicine has developed a variety of biomaterials to assist in the performance and recovery of physiological functions. However, all commonly used implant materials present a constant risk because of bacterial colonization and biofilm formation on their surface. Staphylococci may develop biofilm on central venous catheters or permanently implanted medical devices such as artificial heart valves and surrounding tissues of the heart, leading to a condition known as prosthetic valve endocarditis [15]. As a consequence, biofilm-associated infections can only be resolved by removal of the infected device. This represents a major cost burden on healthcare services.

Strategies focused on disruption and dispersal of established staphylococcal biofilms include activation of the staphylococcal *agr* quorum sensing system [16], use of surfactant-like molecules [17], treatment of biofilms with enzymes that depolymerize biofilm components [18–20] and photodynamic treatment (PDT) [21]. The latter is a process that is based on the combined action of visible light and a photosensitizer drug which generates a cytotoxic reactive oxygen species and free radicals that are bactericidal. In this study, we demonstrate that pretreatment of *S. aureus* biofilms with PDT, followed by addition of vancomycin at concentrations significantly below the biofilm inhibitory concentration (BIC) values, causes disruption of the biofilm matrix and allows an almost complete killing of bacteria. Similar effects were observed when PDT was combined with exposure of disrupted cell clusters to phagocytosis by neutrophils in fresh human blood.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The microorganisms used in this study were *S. aureus* SA113 [22], BH1CC [8] and V329 [6]. *S. aureus* SA113, was a gift from Dr. TJ Foster (Department of Microbiology, Trinity College, Dublin). BH1CC, a MRSA clinical isolate, was a gift from Dr. James O'Gara (UCD School of Biomolecular and Biomedical Science, University College Dublin, Ireland). *S. aureus* V329, a strong biofilm forming isolate obtained from a bovine subclinical phenotype, was kindly donated by Dr. JR Penadés (Universitad Cardenal Herrera-CEU, Moncada, Valencia, Spain). *S. aureus* SA113 and V329 were grown for 16 h aerobically at 37 °C using a shaking incubator (New Brunswick Scientific Co, Edison, NJ) in tryptic soy broth (TSB) (Difco, Detroit, MI). *S. aureus* BH1CC was grown under the same conditions in BHI (Difco).

2.2. Biofilm growth

For biofilm growth, overnight cultures of staphylococci were diluted at 1:200 in TSB (*S. aureus* SA113 and V329) or BHI (*S. aureus* BH1CC) containing 0.25% glucose. Aliquots (200 μl) of the diluted bacterial suspensions were inoculated into 96-well flat-bottom sterile polystyrene microplates (Costar, Corning, Inc. NY) and incubated for 24 h at 37 °C. To enhance *S. aureus* V329 biofilm formation in the assays, microplates were coated with 20% (v/v) human plasma in carbonate buffer (50 mM sodium carbonate, pH 9.5). Bacterial biofilm formation was detected by the method described by Christensen et al. [23]. Briefly, biofilms formed on the plates were washed twice with phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, pH 7.4) to remove planktonic and loosely adhering bacteria [24]. Then, the cells were fixed with 95% ethanol for 10 min and stained with 0.1% crystal violet for 15 min, and after several washings, the wells were air dried. For a quantitative estimation of biofilms, crystal violet was solubilized with 10% glacial acetic acid and absorbance of the solubilized dye was read at 590 nm in a microplate reader (model 680; BioRad Laboratories, Inc., Hercules, CA).

2.3. Enzymatic and chemical treatment of biofilms

Chemical and enzymatic treatment of biofilms were carried out as described previously [25,26]. Briefly, the biofilms grown in microtiter plates were rinsed with 200 μl NaCl (0.9%) and then treated for 2 h at 37 °C as follows: 100 μl sodium metaperiodate (10 mM) (Sigma, St Louis, MO) in 50 mM sodium acetate buffer, pH 4.5; 100 μl proteinase K (Sigma) at 1 mg/ml in 20 mM Tris buffer containing 100 mM NaCl, pH 7.5; 100 μl trypsin (Sigma) at 1 mg/ml in 20 mM Tris buffer containing 100 mM NaCl and 10 mM ethylenediamine tetraacetic acid (EDTA), pH 7.5; 100 μl dispersin B® (Kane Biotech, Inc., Winnipeg, Manitoba) at 40 μg/ml in PBS; 100 μl DNase I (Sigma) at 2 mg/ml in PBS.

For the controls, enzymes or sodium metaperiodate were replaced with the appropriate amounts of buffer.

After the indicated treatment, biofilms were washed with 200 μl NaCl (0.9%), stained with crystal violet and quantified as indicate above. Each condition was evaluated eight times, and each assay was repeated three times.

2.4. Photosensitizer

Tetra-meso (*N*-methyl-pyridyl)porphine (TMP) was kindly supplied by Professor G. Jori (University of Padova, Padova, Italy). The concentration of the porphyrin-type agent was determined by measuring the absorbance at 424 nm in PBS using the molar extinction coefficient $\varepsilon = 194000\ M^{-1}\ cm^{-1}$ [27]. A stock solution of 1 mM TMP was prepared in PBS, filter sterilized and stored at −20 °C. Prior to use, the stock solution was appropriately diluted in PBS to obtain the desired concentration.

2.5. Porphyrin uptake analysis

Bacterial strains were allowed to form biofilm in microtiter wells, as reported above. After washing with PBS, the biofilms were incubated with different porphyrin concentrations (0–15 μM) for 15 min in the dark at 22 °C. To determine the amount of cell-bound porphyrin, the biofilms were suspended and incubated overnight in 2% aqueous sodium dodecylsulfate (SDS). TMP concentration in the cell lysate was determined by measuring the intensity of the porphyrin-typical red fluorescence (600–700 nm) emitted after excitation at 420 nm and interpolating the data on a calibration plot obtained with known porphyrin concentrations. At saturating concentrations, the amount of TMP bound to the cells was 2 pmoles for the strain SA113, 12 pmoles for BH1CC and 9 pmoles for the V329.

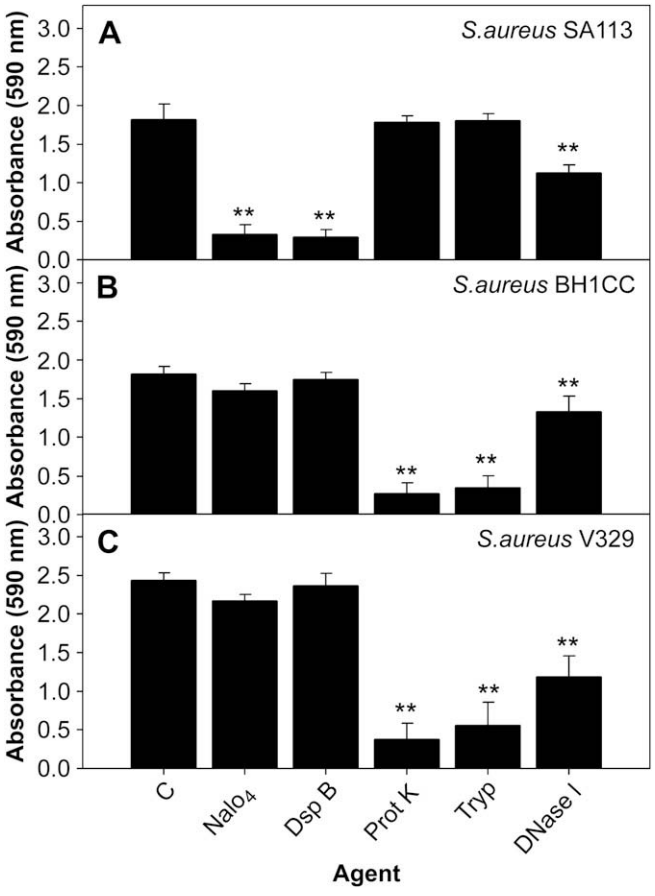

Fig. 1. Effect of enzymatic and chemical treatment on the detachment of *S. aureus* biofilms. Biofilms were grown in 96-well microtiter plates for 24 h at 37 °C and the adherent cells were treated with proteinase K or trypsin, dispersin B, sodium periodate or with DNase I. After washing, adherent cells were stained with crystal violet. The values represent the mean ± standard errors of the two experiments. In each plate, four wells were used for each assay. Asterisks indicate statistically significant decrease in cells attached following enzymatic digestion or chemical treatment compared to the untreated biofilm.