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A mucosal model to study microbial biofilm development and anti-biofilm therapeutics

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ABSTRACT

Biofilms are a sessile colony of bacteria which adhere to and persist on surfaces. The ability of bacteria to form biofilms is considered a virulence factor, and in fact is central to the pathogenesis of some organisms. Biofilms are inherently resistant to chemotherapy and host immune responses. Clinically, biofilms are considered a primary cause of a majority of infections, such as otitis media, pneumonia in cystic fibrosis patients and endocarditis. However, the vast majority of the data on biofilm formation comes from traditional microtiter-based or flow displacement assays with no consideration given to host factors. These assays, which have been a valuable tool in high-throughput screening for biofilm-related factors, do not mimic a host–pathogen interaction and may contribute to an inappropriate estimation of the role of some factors in clinical biofilm formation. We describe the development of a novel ex vivo model of biofilm formation on a mucosal surface by an important mucosal pathogen, methicillin resistant *S. aureus* (MRSA). This model is being used for the identification of microbial virulence factors important in mucosal biofilm formation and novel anti-biofilm therapies.

1. Introduction

Biofilms are communities of micro-organisms that are embedded in an extracellular matrix composed of proteins, lipids, polysaccharides and nucleic acids. The members of a biofilm are protected from environmental factors such as UV light and dehydration, as well as from host immune cells such as neutrophils and other phagocytes (Hall-Stoodley et al., 2004). Biofilm associated bacteria are also much more resistant to antimicrobial agents (Stewart and Costerton, 2001).

Recently there has been a great deal of interest in the role of biofilms in infectious diseases. The National Institutes of Health has estimated that ~80% of human infections are caused by pathogenic biofilms, (SBIR/STTR study and control of microbial biofilms, http://grants.nih.gov/grants/guide/pa-files/PA-99-084.html). The sites of biofilm-mediated infections include temporary or permanent medical devices (endotracheal tubes, intravascular and urinary catheters, orthopedic implants and arterial stents) and wounds, blood, and mucosal surfaces (sinuses, respiratory and genito-urinary tracts) (Bakaletz, 2007; Donlan, 2001; Wolcott et al., 2008).

The mucosa represents a surface where microbes from the external environment interact with host tissues (Kaufmann et al., 2011). Often, these interactions are beneficial or benign, as in the case of commensal organisms. However, the mucosa remains a major site of entry for pathogens. Barrier function alone is often insufficient in protecting against microbial pathogens. Therefore, the mucosa also contains cells and soluble regulatory and effector molecules of the innate and adaptive immune systems (Janeway and Medzhitov, 2002). These constituents include epithelial cells, neutrophils, macrophages, dendritic cells, natural killer cells, T and B lymphocytes, mucin and a variety of effector peptides and proteins such as defensins, complement, C-reactive protein, and pro-inflammatory chemokines and cytokines (Kaufmann et al., 2011). These serve as critical components of the host immune response to infections.

The study of biofilm formation on biologically relevant surfaces, such as mucosal tissue, allows for a clearer understanding of host/pathogen interactions as the model system used can have profound effects on the expression of potential virulence factors involved in biofilm formation (Costerton et al., 1987; Otto, 2008). In fact, it has been demonstrated that the substrate used for biofilm formation can impact bacterial gene expression (Anderson et al., 2008; de Breij et al., 2009). These studies underscore the importance of using a biological matrix for studies of medical biofilm growth that will inform the development of treatments for clinical infections.

In vivo studies are the gold standard in pathogenesis, but they are expensive and labor intensive. Co-cultures of pathogens and mammalian cell lines, while contributing valuable information, can only be conducted over short periods of time (i.e. less than 24 h). This is due to the cytotoxic effects of the pathogens on the cells, which reduces the utility of these studies as biofilms can take multiple days to reach maturity. For example, it was recently shown U2OS osteosarcoma cells infected with *S. aureus* or

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Pseudomonas aeruginosa rounded up and detached by 18 h post-infection, however, growth of both the U2OS cells and the less virulent, commensal organism *S. epidermidis* was observed as late as 48 h (Subbiahdoss et al., 2011). Reconstituted human epithelial tissue cultures have been used as a surrogate for in vivo *C. albicans* biofilm studies, but the construction of the 3-dimensional tissue culture is also labor intensive and expensive. Recognizing these limitations, we developed an ex vivo full thickness tissue model to study biofilm formation by clinical isolates of MRSA, an important pathogenic bacteria.

The human vaginal surface is comprised of non-keratinized, stratified, squamous epithelium and interspersed cells of the immune system covered by a layer of mucus (Thompson et al., 2001). *S. aureus* commonly colonizes mucosal surfaces such as the vagina and the anterior nares (Lowy, 1998). Colonization has been associated with an increased risk of *S. aureus* infections (Kluytmans et al., 1995). Up to 10% of women are colonized vaginally with *S. aureus* (Martin et al., 1982) and as many as 20–60% of people are colonized in the anterior nares persistently and transiently, respectively (Kluytmans et al., 1997).

We selected porcine vaginal mucosa (PVM) as our *S. aureus* biofilm model substrate for multiple reasons. Most infections initiate at mucosal surfaces and *S. aureus* colonizes the human vaginal mucosa. Like its human counterpart, PVM consists of stratified, squamous epithelium, protected by a layer of mucus (Kong and Bhargava, 2011; Squier et al., 2008). The porcine vagina is relatively large in size, so that many small biopsies may be obtained from a single specimen, which in turn allows us to test multiple variables in our experiments without concerns of inter-animal variability. It is inexpensive and easy to procure. Finally, the culture of a stratified, squamous epithelium and underlying connective tissue allows us to study mature biofilm formation over the course of 3 days.

Here we present a novel mucosal biofilm model, which enables the study of biofilm formation and the factors that contribute to a biofilm phenotype by MRSA, in an environment mimicking a natural infection. This model can also be utilized as a semi-high throughput platform for novel anti-biofilm therapy development, as not only can we assess efficacy, but also host cytotoxicity. We used the MRSA mucosal biofilm model to demonstrate a lack of efficacy by 0.12% chlorhexidine gluconate (CHG, Peridex™) on biofilm formation. CHG is an antiseptic agent commonly used in oral rinses within the dental community and more recently for prevention of ventilator-associated pneumonia (VAP). CHG has also been used to decolonize the vagina prior to delivery for prevention of transmission of β–hemolytic *Streptococcus* as well as treatment of vaginal infections in non-pregnant women, at concentrations ranging from 0.25 to 2% (Goldenberg RL et al., 2006; Molteni et al., 2004; Wilson et al., 2004).

2. Methods

2.1. Tissue preparation and bacterial culture

Normal healthy porcine vaginal tissue was excised from animals at slaughter (Andrew Boss Laboratory of Meat Science, University of Minnesota, St. Paul, MN) and transported to the laboratory in RPMI 1640 media (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (Invitrogen, Carlsbad, CA), penicillin (50 IU/mL, MP Biomedicals, Solon, OH), streptomycin (50 mg/mL, MP Biomedicals, Solon, OH) and amphotericin B (2.5 μg/mL, Hyclone, Logan, UT). Antibiotics were included to decolonize normal flora which may interfere with biofilm formation. Tissue was utilized within 3 h of excision. Explants of uniform size were obtained from the porcine vagina using a 5 mm biopsy punch. Excess muscle tissue was trimmed away with a scalpel. Tissue explants were washed in antibiotic-free RPMI 1640 media 3 times. The explants were then placed mucosal side up on a PET track-etched 0.4 μm cell culture insert (BD Bioscience, Franklin Lakes, NJ) in 6-well plates containing fresh serum-free and antibiotic-free RPMI 1640 and incubated at 37 °C. The mucosal surface was continually exposed to air.

A biofilm forming bioluminescent strain of MRSA (Xen30, parent strain is a clinical isolate from Roche) was purchased from Caliper Life Sciences (Caliper Life Sciences, Hopkinton, MA). This isolate was selected for its previously characterized capability to form robust biofilms in vitro and in vivo. Stationary phase (overnight) cultures of MRSA were washed in RMPI 1640 and resuspended to a concentration of approximately 5×10^8 CFU/mL. Two microliters of this suspension was then used to inoculate tissue explants on the mucosal surface (1×10^6 CFU/explants). Explants were returned to 37 °C and incubated for 0–72 h.

2.2. MTT evaluation of tissue viability

Viability of the explants was quantified by the reduction of the tetrazolium salt 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT) by live tissue into an acidified isopropanol soluble product (CGDA kit, Sigma, St. Louis, MO). Briefly, after culturing for the indicated amount of time, explants were transferred to 96-well tissue culture plates containing 100 μl serum-free, antibiotic-free RPMI 1640 and 10 μl MTT substrate. The explants were returned to the 37 °C incubator for 3 h. After incubation, the purple formazan product was extracted using acidified isopropyl alcohol at 4 °C for 18 h. Optical density in the wells was measured following removal of explants. Data are expressed relative to fresh tissue which was used as the maximum viability control.

2.3. Enumeration of CFU

Bacteria were enumerated from infected explants by homogenization (highest setting for 30 s) or vortex mixing (highest setting, 2 m) in 250 μl sterile phosphate buffered saline (PBS, Sigma, St. Louis, MO) or CHG neutralizing solution containing Triton X-100 (Aldrich, St. Louis, MO), lecithin (Alfa Aesar, Ward Hill, MA), Tween 80 (Sigma, St. Louis, MO) and sodium thiosulfate (Sigma, St. Louis, MO). Homogenates/supernatants were then serially diluted in PBS (or plated neat) and spread on Tryptic Soy Agar containing 5% sheep's blood (Becton Dickenson, Franklin Lakes, NJ) using a spiral plater (Biotek, Microbiology International, MD). In some experiments, explants were gently washed 3× in PBS prior to vortex mixing to count planktonic vs. adherent (biofilm) bacteria.

2.4. Scanning electron microscopy

Infected and control explants were washed gently in normal saline (Becton Dickenson, Franklin Lakes, NJ). They were then fixed and processed as previously described (Erlandsen et al., 2004). Briefly, explants were fixed in 2% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) + 2% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA) + 0.15% alcian blue (Sigma-Aldrich, St. Louis, MO) in 0.15 M cacodylate buffer for 22 h. Specimens were washed in 0.15 M cacodylate (Electron Microscopy Sciences, Hatfield, PA) buffer and then post-fixed in 1% OsO_4 (Electron Microscopy Sciences, Hatfield, PA) + 1.5% potassium ferrocyanide (Sigma-Aldrich, St. Louis, MO) in 0.15 M cacodylate buffer for 90 m, protected from light. Specimens were again washed 3× in 0.15 M cacodylate buffer, then dehydrated in an ethanol series: 50%, 70%, 80%, 95% and 2× 100% for 5 m per step. After critical point drying in CO_2, specimens were mounted mucosal side up on SEM stubs using adhesive carbon tabs (Ted Pella, Redding, CA). Specimens were sputter coated with approximately 1–2 nm platinum and imaged on a Hitachi S-4700 field emission SEM at 2 keV.

2.5. Laser scanning confocal microscopy

Infected and control explants were stained using FilmTracer™ LIVE/DEAD® Biofilm Viability kit (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. After staining, specimens were gently