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Research Paper

Bacterial Abscess Formation Is Controlled by the Stringent Stress Response and Can Be Targeted Therapeutically

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ABSTRACT

Cutaneous abscess infections are difficult to treat with current therapies and alternatives to conventional antibiotics are needed. Understanding the regulatory mechanisms that govern abscess pathology should reveal therapeutic interventions for these recalcitrant infections. Here we demonstrated that the stringent stress response employed by bacteria to cope and adapt to environmental stressors was essential for the formation of lesions, but not bacterial growth, in a methicillin resistant *Staphylococcus aureus* (MRSA) cutaneous abscess mouse model. To pharmacologically confirm the role of the stringent response in abscess formation, a cationic peptide that causes rapid degradation of the stringent response mediator, guanosine tetraphosphate (ppGpp), was employed. The therapeutic application of this peptide strongly inhibited lesion formation in mice infected with Gram-positive MRSA and Gram-negative *Pseudomonas aeruginosa*. Overall, we provide insights into the mechanisms governing abscess formation and a paradigm for treating multidrug resistant cutaneous abscesses.

1. Introduction

Abscesses are very common. For example, in the United States alone, 3.2 million people were treated in hospital emergency departments for an abscess infection in 2005 (Taira et al., 2009). Cutaneous abscesses, triggered by local bacterial infections, are characterized by an accumulation of fluid/pus within the dermis, which is often associated with severe inflammation and induration and frequently lead to a skin lesion that may present as an open sore (Singer and Talan, 2014). Severe abscesses that display signs of septic infection are not only surgically drained, but also treated with antibiotics to prevent dissemination, although recurrence can occur (Singer and Talan, 2014). An example of a bacterium that often causes abscesses is Methicillin-resistant *Staphylococcus aureus* (MRSA). In addition to its role as an important nosocomial human pathogen, MRSA infections are now emerging in the community as an important cause of skin and soft-tissue infections, many of which are cutaneous abscesses (Moran et al., 2006). Common antibiotic therapies for MRSA abscesses include trimethoprim-sulfamethoxazole, clindamycin and tetracyclines (Singer and Talan, 2014; Talan et al., 2011). However, conditions within the abscess such as low pH, excessive debris and high bacterial loads, as well as low redox potential, have been shown to limit the penetration and efficacy of antibiotics (Stearne et al., 2001). Thus antibiotics work poorly against abscess infections and the substantial consumption of antibiotics over the past decades has resulted in strains that are resistant to virtually all of the utilized antibiotic treatments (Moran et al., 2006; Singer and Talan, 2014; Talan et al., 2011). Cutaneous abscesses can also be caused by a variety of other Gram-negative (Carpenter, 1990) and Gram-positive bacteria (Maliyil et al., 2011) making broad spectrum treatments more desirable.

Here we hypothesized that abscesses represent a distinct stress-triggered growth state that make them both resistant to treatment and able to cause pathology. In particular, we have considered the involvement of the universal stringent stress response. The stringent response is a conserved stress response employed by various bacteria to respond to and cope with conditions of amino-acid starvation, carbon-source, fatty acid, oxygen or iron limitation, heat shock, antimicrobial challenge, and/or other environmental stressors (Crosse et al., 2000; Potrykus and Cashel, 2008). In most bacteria, the stringent response is signaled by the secondary-messenger guanosine tetraphosphate (ppGpp), which serves as a pleiotropic transcriptional regulator by binding to RNA polymerase (Dalebroux and Swanson, 2012). This leads to the repression of resource-consuming processes (translation, lipid, and cell wall biosynthesis, and to some extent replication and transcription) and diverts resources toward biosynthesis (amino acid biosynthesis and transport, glycolysis) and diverse stress genes to promote survival (Potrykus and Cashel, 2008; Srivatsan and Wang, 2008; Wolz et al., 2010).

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Cationic amphipathic peptides are an evolutionarily conserved, multifunctional component of the innate immune system. They are known to have immunomodulatory, direct antimicrobial, and/or anti-biofilm activity (Hancock and Sahl, 2006). Importantly, a distinct subset of cationic peptides have demonstrated broad-spectrum efficacy in targeting recalcitrant biofilm infections by targeting the stringent stress response (de la Fuente-Núñez et al., 2014; de la Fuente-Núñez et al., 2015; Pletzer and Hancock, 2016). Biofilms are a distinct growth state of bacteria on surfaces whereby the bacteria form structured aggregates that are adaptively multi-antibiotic resistant (de la Fuente-Núñez et al., 2014; de la Fuente-Núñez et al., 2015; Overhage et al., 2008). The stringent response and biofilm formation are tightly interconnected processes since ppGpp is required for biofilm initiation and maintenance, such that bacterial mutants defective in the stringent response do not form biofilms (Aberg et al., 2006; de la Fuente-Núñez et al., 2014; He et al., 2012). In *S. aureus*, upon amino acid starvation, ppGpp (and its precursor pppGpp) production is mediated by the bi-functional synthase/hydrolase enzyme RSH (a RelA/SpoT homolog) (Geiger et al., 2012).

The ppGpp regulon is very complex (Vercruysse et al., 2011). For example, in *Escherichia coli*, ppGpp mediates the induction of other stress regulators within the universal stress protein (USP) family (Kvint et al., 2003). Likewise, in *S. aureus*, a homolog, the universal stress protein (designated Usp2) was recently identified and shown to be necessary for persistence under amino acid starvation (Attia et al., 2013) and is positively regulated by ppGpp (Geiger et al., 2012). Despite these findings, the importance of these stringent response regulators in *S. aureus* pathogenesis remains an understudied topic.

Here we have demonstrated a contribution of the stringent response to *S. aureus* cutaneous abscess formation (as judged by lesion formation and other altered pathology findings), but not local bacterial growth, and demonstrated that it can be targeted pharmacologically with a peptide. Furthermore, the same pharmacological targeting worked with *P. aeruginosa* infections in a cutaneous abscess model.

2. Materials and Methods

2.1. Strains

S. aureus wild-type HG001, RSH synthase mutant (rsh_{syn}) and complemented RSH synthase strain were kindly provided by Christiane Wolz (University of Tübingen, Tübingen). *S. aureus* Newman and Δ*usp2* were provided by Eric Skaar (Vanderbilt University Medical Center, Nashville, TN). Flow cell analysis was conducted on biofilm forming MRSA strain SAP0017 (clinical isolate kindly provided by Dr. Tony Chow, Vancouver General Hospital). For in vivo peptide studies, bioluminescent *S. aureus* USA300 was used and kindly provided by Scott Stibitz (Food and Drug Administration, Silver Spring, MD) and *P. aeruginosa* LESB58 (Liverpool epidemic strain) was from Winstanley et al. (Winstanley et al., 2009). All strains in Table S1 were kindly provided by Michael Otto (National Institute of Health, Bethesda, MD).

2.2. Peptide Synthesis

DJK-5 (VQWRAIRVRVIR-NH_2; all D amino acids) was synthesized by CPC Scientific and control peptide IDR-2013 (WQRVRRVKVIRK-NH_2) was synthesized by Genscript using solid-phase 9-flurenylmethoxy carbonyl (Fmoc) chemistry and purified to >95% purity using reverse-phase high-performance liquid chromatography (HPLC). The lyophilized peptide was initially resuspended in endotoxin-free water and used in vitro, or further resuspended in saline and used in vivo.

2.3. Drug Susceptibility Test

The broth microdilution method with minor modifications for cationic peptides (Wiegand et al., 2008) was used for measuring the MIC of peptide DJK-5.

2.4. Flow Cell Analysis

A flow cell system was initially assembled and sterilized as previously described (de la Fuente-Núñez et al., 2014). BM2 biofilm-adjusted medium [62 mM potassium phosphate buffer (pH 7), 7 mM $(NH4)_2SO_4$, 2 mM $MgSO_4$, 10 μM $FeSO_4$, 0.4% (wt/vol) glucose] was allowed to pump through the flow cell apparatus for 1 h before the chambers were injected with 1/20 dilutions of overnight culture and bacteria allowed to adhere to the plastic surface of the flow cells for 3 h. To assess the activity of the peptide on pre-formed biofilms, peptide was added to the system two days after the initial bacterial injection and pumped through the system for a subsequent 24 h. Three days following the injection, the flow cells were injected with SYTO-9 and propidium iodide stain [LIVE/DEAD BacLight Bacterial Viability kit (Molecular Probes, Eugene, OR)] to image total and dead cells respectively. Remaining biomass was assessed using a confocal laser scanning microscope (Olympus, Fluoview FV1000) and three-dimensional reconstructions were generated using the Imaris software package (Bitplane AG).

2.5. Mouse Skin Infection Model

Female CD-1 mice (6 weeks old) were purchased from Charles River Laboratories (Wilmington, MA) and were used for the abscess model. Mice were housed together (maximum five/cage) and bedding (shredded paper) and nestlets were provided in each cage. All animal experiments were performed in accordance with The Canadian Council on Animal Care (CCAC) guidelines and were approved by the University of British Columbia Animal Care Committee. The fur on the backs of the mice was removed through shaving and depilatory cream. All *S. aureus* strains were grown to an optical density at 600 nm (OD_{600}) of 1 in tryptic soy broth (TSB), while *P. aeruginosa* LESB58 was grown in double yeast tryptone (dYT) medium to an OD_{600} of 1; subsequently cells were washed twice with sterile PBS, and resuspended to a final concentration of 5×10^7 CFU / 50 μL. For mutant studies, bacteria were injected into the right flank of the back. For peptide intraperitoneal (IP) administration studies, mice were initially given either saline or 6 mg/kg DJK-5 (for MRSA studies) or 4 mg/kg DJK-5 (for *P. aeruginosa* studies) in saline via IP injection immediately before applying 50 μL of bacteria subcutaneously to the right flank of the back. For intra-abscess studies, mice received 50 μL of bacteria and 1 h later, 3 mg/kg of peptide via intra-abscess injection. Abscess lesion sizes were measured using a caliper every 24 h for a maximum of 5 days. Visible dermonecrosis or white lesions (filled with pus) were considered as part of the abscess lesion. Swelling/inflammation was however disregarded in the measurements. Mice were monitored once daily and no adverse outcomes were reported. To assess the levels of luminescent bacteria in the abscess every 24 h, the in vivo imaging System (IVIS) (Perkin Elmer, Waltham MA) was utilized. Skin abscesses were excised either two, three or five days post-infection, homogenized using a rotor stator for 5 min and serially diluted for CFU quantification. Furthermore, skin explants were fixed in 10% neutral buffered formalin and processed for hematoxylin and eosin and Gram staining using a Wax it kit (University of British Columbia).

2.6. Evaluation of Histological Slides

Histological and Gram stained slides were independently evaluated blindly by veterinarian Dr. Ian Welch at the Centre for Comparative Medicine (Vancouver, Canada) and pathologist Dr. Hamid Masoudi at Vancouver Coastal Health (Vancouver, Canada). All slides used for this independent evaluation are available from the authors upon request with one being presented in Results.

2.7. Measurement of Phenol Soluble Modulins (PSM)

For luminescent reporter studies, the USA300 PSMα luminescence reporter strain (Dastgheyb et al., 2015) was grown in TSB in the

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