Early effects of *Staphylococcus aureus* biofilm secreted products on inflammatory responses of human epithelial keratinocytes

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Abstract

Chronic wounds such as diabetic foot ulcers, pressure ulcers, and venous leg ulcers are a leading contributor toward mortality throughout the world. The inability of these wounds to heal is associated with the presence of microbial biofilms. The objective of this study was to determine if products secreted by *Staphylococcus aureus (S. aureus)* biofilms play an active role in chronic wounds by promoting inflammation, which is a hallmark of chronic wounds.

In vitro experiments were conducted to examine changes in gene expression profiles and inflammatory response of human epithelial keratinocytes (HEKa) exposed to products secreted by *S. aureus* grown in biofilms or products secreted by *S. aureus* grown planktonically.

After only hours of exposure, gene expression microarray data showed marked differences in inflammatory, apoptotic, and nitric oxide responses between HEKa cells exposed to *S. aureus* biofilm conditioned media (BCM) and HEKa cells exposed to *S. aureus* planktonic conditioned media (PCM). As early as 4 hours post exposure, enzyme linked immonosorbant assay (ELISA) results showed significant increases in interleukin 6 (IL-6),interleukin 8 (IL-8), tumor necrosis factor alpha (TNFα), and chemokine ligand 2 (CXCL2) production by HEKa cells exposed to BCM compared to HEKa cells exposed to PCM or controls. Nitric oxide assay data also showed significant increases in nitric oxide production by HEKa cells treated with BCM compared to HEKa cells treated with PCM, or controls. These results support and extend previous findings that indicate products secreted by *S. aureus* biofilms directly contribute to the chronic inflammation associated with chronic wounds.

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Chapter 1: Introduction

Wound Healing

Skin is the primary barrier between the body and the outside world. When a break in the skin or skin wound occurs it is imperative that it heals in a timely and efficient manner to minimize the ability for pathogenic bacteria to colonize [Stadlemann, 1998]. Skin wounds fall in to two categories, acute and chronic. Acute wounds heal in an uncomplicated manner and follow an orderly pattern of healing. Chronic wounds can be defined in a variety of ways, but the most common definition is a wound that takes an extended time to heal or does not heal in an orderly fashion using standard therapy [Fletcher, 2008]. Most commonly, if a wound has not healed in 4-6 weeks it is considered a chronic wound [Siddiqui and Bernstein, 2010].

Acute wounds heal quickly, and in a conventional pattern with little or no fibrosis [Stedelmann, 1998]. This pattern of healing is well-defined and includes: inflammation, proliferation, contraction and tissue remodeling [Dieglemann, 2004]. The first phase in the healing process is the inflammatory phase. For approximately the first 4 days after injury there is an increased blood supply to the wound site that brings in fibronectin, polymorphonuclear leukocytes, and mononuclear leukocytes which mature into macrophages and then lymphocytes. The fibronectin that is deposited in the wound site forms a fibrin clot which serves as the matrix that supports the rest of the healing process [Stadelmann et al., 1998]. Fibroblasts begin constructing the extracellular matrix by synthesizing a provisional extracellular matrix that is subsequently replaced by collagen fibers [Epstein, 1999]. This fibrin clot also provides the scaffolding in which fibroblasts migrate to the wound site [James et al., 2008]. After the creation of the fibrin clot the keratinocytes surrounding the wound start to produce chemical signals in the form of cytokines and chemokines [Stadelmann et al., 1998]. These signals attract neutrophils and monocytes to the wound site. Neutrophils play an important role in the healing process by phagocytizing bacteria that may try to colonize the wound site as well as serve as an early source of pro-inflammatory cytokines that serve as signals to activate fibroblasts. While neutrophils are signaling cytokines macrophages are also rushing to the site of the wound. Macrophages rush to the site of the wound in order to phagocytize the

pathogenic microorganisms as well as clearing away any other cell matrices or debris that might be found in the site of the wound [James et al., 2008; Serralta et al., 2001].

After the inflammatory phase comes the proliferative phase that can last up to two years [Thomas and Harding, 2002]. This phase involves the production of TGF-β which increases the transcription of genes responsible for collagen, proteoglycans and fibronectin as well as increasing the overall production of matrix proteins. TGF-β also decreases proteases responsible for the breakdown of the matrix [Thomas and Harding, 2002]. Fibroblasts are stimulated by TGF-β1 and other growth factors and completely invade the wound about a week after the fibrin clot forms [Darby et al.,1990]. Fibroblasts produce collagen that forms the lattice that will eventually lead wound closure [Stadelmann, 1998]. A portion of fibroblast transform into myofibroblasts that express α-smooth muscle actin that resembles smooth muscle that generates strong contractile fibers [Darby and Gabbiana, 1990]. Keratinocytes play a role in the modulation of fibroblasts so that they can transition from deposition of collagen and other elastic fibers to a phenotype that functions more in the deposition of ECM and there functions more in the remodeling phase [Menon et al., 2012]. This remodeling phase of the healing process stimulates the crosslinking of collagen fibers in order to from scar tissue [James et al., 2008].

Remodeling is the last phase of the acute healing process and allows the wound to form some lasting scar tissue. The collagen fibers that have been formed in previous phases reorganize to produce the scar tissue and the final epithelial tissue that ends the healing process [Serralta, 2001]. Even with the relative organization of this process acute wound healing can have a variety of outcomes and timelines. The amount of scar tissue can vary widely. In wounds that have excessively long healing rates, hypertonic scarring occurs through the excessive production of collagen [Menon et al., 2012].

Wounds that exhibit delayed healing or a failure to heal are called chronic wounds. Chronic wounds can occur in the form of diabetic ulcers, pressure sores as well as venous and arterial ulcers. These wounds often come with a variety of issues including impaired granulocytic function, impaired chemotaxis, and an increase in the inflammatory phase, impaired neurovalsculation, decreased synthesis of collagen, increased levels of proteinases, and defective macrophage function [Epstein, 1999]. During the wound healing process granulation tissue provides a rich bed of capillaries which provide a matrix for

inflammatory cells, macrophages, and fibroblasts. In chronic wounds the creation of this matrix is impaired which leads to a delay in healing [Stadelmann, 1998].

Bacterial colonies are also a common feature of chronic wounds. These colonies create increased inflammation, which increases neutrophil and macrophage migration to the site. This migration stimulates inflammatory cytokines and degrades the extracellular matrix, which causes tissue hypoxia and leads to slower or an absence of wound healing at the site [Newton and Dixit, 2012].

Bacterial Biofilms

Bacteria have the ability to grow in two forms: planktonically and in biofilms form. Planktonic bacteria live as single cells that are motile and have little or no communication with other bacterial cells [Landi et al., 2009]. Biofilms are bacteria that have converted to a sessile form and adhere to a surface. Biofilms can result from a variety of environmental cues and consist of multilayers of bacterial cells adherent to a biotic or abiotic surface [Chaignon et al. 2007]. The transformations from the planktonic to sessile form, and along with the production of polysaccharide intracellular adhesions, are important for the formation of bacterial biofilms [Cramton et al., 1999].

When bacteria form biofilms they alter their physiology, gene expression and morphology [Landi, 2007]. The first step to biofilm formation is the adherence of the bacterial cells to a surface. Bacterial biofilms seem to grow most readily on surfaces that are impaired due to systemic disease (like diabetes or arthritis) or local factors (arterial insufficiency or venous hypertension). These damaged areas provide a fertile bed for bacterial colonization that can ultimately lead to the formation of bacterial biofilms [Edwards and Harding, 2004]. The contact with a favorable surface leads to a series of gene expression changes that promote a more stable connection between the bacterial cell and surface [Stanley, 2004]. Two of the genes that have been found to play a role in the transformation of S. *aureus* into its sessile form are *ica* and *sarA* [Beeken et al., 2003; Cramton et al., 2001]. After conversion of its sessile form bacterial biofilms must adhere to the surface. *Bap*, which codes for a surface protein, has been shown to play a part in the mediation of this initial adhesion of *S. aureus* to a surface [Cucarella et al., 2001].

After cellular adhesion is the formation of the bacteria micro-colony. Micro-colonies can be several layers thick and can be composed of a single species of bacteria or multiple species that depend on cell-cell interactions [Stanley, 2004]. The adhesion of bacterial cells in multiple layers is followed by the creation of a polysaccharide intercellular adhesion (PIA) portion of the extracellular matrix (ECM) [Gotz, 2002]. PIA links bacterial cells together and may play a role in the adherence of biofilms to surfaces [Gotz, 2002].

Another important factor to the production and maintenance of bacterial biofilms is quorum sensing. Quorum sensing bacteria produce and release chemical signal molecules called autoinducers that lead to an alteration of gene expression. This alteration allows bacterial cells to secrete chemical signals that act as a communication network between the bacterial cells in the micro-colony matrix [Yarwood, 2004]. This communication network is essential to biofilm formation and the ability of bacteria to resist antibacterial properties in wounds and other natural environments [Landini, 2009; Miller and Bassler, 2001]. In gram-positive cells like *S. aureus* the bacterial cells communicate using modified oligopeptides as signals and a "two-component" type membrane bound sensor histidine kinases as receptors. Signaling is mediated through a response regulator which creates a circuit that mediates intraspecies communication [Waters and Bassler, 2005]. This circuit is created by a phosphorylation cascades that influences the activity of a DNA-binding transcriptional regulatory protein termed a response regulator [Landini, 2009]. *agr* has been found to be upregulated in *S. aureus* that grown in biofilms. *agr* has been found to play a role in the secretion of chemical signals that are associated with the creation of the polysaccharide matrix and quorum sensing which are essential to the functioning of bacterial biofilms [Schierle et al., 2009].

In order to create a bacterial biofilm that would allow us to investigate the genomic and inflammatory changes that occur between keratinocytes exposed to planktonic cells and bacterial biofilms we used *S. aureus. S. aureus* was chosen because it is the most common bacteria found in chronic wounds infected with bacterial biofilms and has been used in other studies on chronic wounds [Schierleet et al, 2009]. These earlier studies have found that *S. aureus* biofilms prevent re-epithelialization and thus play a role in the proliferation and persistence of chronic wounds [Schierleet et al., 2009].

S. aureus typically is a commensal organism that is a common part of the normal skin flora of humans. But, in the presence of a wound or break in the skin *S. aureus* can colonize the area and create infection [Resch et al., 2005]. A key part of biofilm formation is the creation of the polysaccharide intracellular adhesion as part of a polysaccharide matrix which is composed of β-1, 6-linked *N*-acetoglucosimine with partially acetylated residues. The polysaccharide matrix is an essential part in the ability of *S. aureus* biofilms to resist treatment with antibiotics as well as the host immune response [Resch, 2004].

S. aureus and wounds

S. aureus is a gram-positive pathogen that can cause a variety of illnesses from food poisoning, endocarditis, and toxic shock syndrome and skin illnesses. *S. aureus* along with *S. epidermis* is one of the most isolated organisms in acute and chronic skin wounds. Typically, *S. aureus* is a commensal organism that is present persistently in 30% of the population [Massler and Bassler, 1999]. Often *S. aureus* is carried in the nasal passages and a person's role as a carrier of *S. aureus* is a significant risk factor in whether a person acquires a staph infection [Wertheim et al., 2005]. In the absence of some break in the skin *S. aureus* carriers are asymptomatic and show no disease. The pathogenic nature of *S. aureus* does not occur until the pathogen finds some route into the bloodstream where it can create a number of problems for the host organisms [Archer, 1998].

The type and severity of *S. aureus* infection varies greatly depending on the virulence factors involved. *S. aureus* pathogenicity is greatly determined by unique forms of virulence genes that exist in that clone or lineage [Jarraud et al., 2002]. *S. aureus* pathogenicity is related to several components found on the surface as well as extracellular proteins. (1) Surface proteins that promote colonization of host tissues. (2) Factors that inhibit phagocytosis (capsule, immunoglobulin binding protein A). (3) Toxins that damage host tissues and cause disease symptoms. Coagulase-negative staphylococci are normally less virulent and express fewer virulence factors. *Staphylococcus epidermis (S. epidermis)* readily colonizes implanted devices [Baron, 1996].

In order for the *S. aureus* pathogen to cause disease it contains several factors that allow it to adhere to host cells. *S. aureus* surface proteins attach to host proteins such as laminin and fibronectin that are a part of the extracellular matrix [Baron, 1996]. A surface protein that is often associated with cutaneous skin infections is Panton-Valentine leukocidin. This protein damages the membranes of the host defense cells and erythrocytes [Lina et al., 1999]. Another protein that has been implicated in the ability of *S. aureus* to cause infection and illness is peptidoglycan. Peptidoglycan has been shown to induce the release of inflammatory cytokines in host cells, induce polyclonal activation of T-cells, and cause organ injury when it is combined with lipoteichoic acid (LTA) and lipopolysaccharide (LPS) [Wang et al., 2004].

Once the *S.aureus* pathogen has colonized the host it must avoid the host defenses in order to propagate itself. One of the ways that *S. aureus* avoids elimination in the host is by inhibiting phagocytosis through Protein A [Mempel et al., 1998]. Protein A binds to immunoglobin G by the fragment crstallizable (Fc) region and disrupts phagocytosis and opsonization [Baron, 1996]. Protein A also allows *S. aureus* to facilitate adhesion to human epithelial cells [Mempel et al., 1998]. It has been found to attach to the surface of endothelial cells and stimulate and immune response which includes the stimulation of TNF-α. TNF-α is an important immune regulator and can induce fever, stimulate immune cells and induce apoptosis. These immune responses can lead to more severe infection allowing the bacterium to present a more invasive disease path [Edwards et al., 2012]. Capsular polysaccharides are also on the surface of the *S. aureus* cell. These capsular polysaccharides increase the virulence of *S. aureus* by making the bacterial cells more resistant to phagocytosis [O'Riordan and Lee, 2004].

The last way that *S. aureus* is able to circumvent host defenses is through damaging the membranes of the host cells. *S. aureus* has several toxins that given the right host receptor can adhere specific cell receptors and create gaps or pores in the host cells and can lead to sepsis and toxic shock in affected hosts [Peacock et al., 2002]. Synergohymenotropic toxins damage membranes of host defense cells and erythrocytes. These toxins are found in more than 99% of *S. aureus* strains and provide the bacterial cell with a way to damage host defense and thus propagate and grow [Lina et al., 1999]. Expression of most of the virulence factors described above is controlled by the *agr* locus. Analysis of the genetics of the *agr* locus reveals that specific *agr* clones produce different virulence factors and thus

produce different disease paths [Jarraud et al., 2002]. This contributes to the overall ability of *S. aureus* to resist host defense and establish and maintain colonization.

Another key to the ability of *S. aureus* to infect in a variety of ways is the presence of pathogenicity islands. These parts of the genome carry a high number of genes that are responsible for virulence factors which lead to disease in humans and animals [Lindsey and Holden, 2004]. The variety of virulence factors is a key factor to the ability of *S. aureus* to cause a variety of skin infections. One such infection is scalded skin syndrome or Ritter disease. This type of infection is stimulated by exfoliative toxin and causes blisters on human skin [Yamaguchi et al., 2002]. The main role of exoproteins is to convert the host tissues into nutrients needed for bacterial growth [Dinges et al., 2000]. As a whole many factors play a role in the ability of *S. aureus* to cause disease and resist the host immune system. Growth in the form of a biofilms is one of those devices [Stewart and Costerton, 2001]. The quorum-sensing system regulates which toxins and virulence factors are produced in *S. aureus* biofilms. This system allows the biofilm to better evade host defenses that the bacteria does in planktonic form [Costerton et al., 2007].

Keratinocytes

Skin has three layers; a top layer of a keratinized stratified epidermis, a collagen rich dermal layer and a subcutaneous layer. In unwounded skin a basal layer of keratinocytes attaches to the basal lamella. When a cutaneous wound occurs, the top layer of skin is disrupted and colonization of bacteria can occur. This colonization can lead to infection that creates significant morbidity and mortality for patients [Beckrich and Aronovitch, 1999 Boyko et al. 1996, Landi et al. 2007]. Keratinocytes must form a monolayer through migration and only then can the underlying basal layer of stratified epidermis be formed [Martin, 1997]. In normal wound healing collagenase is expressed consistently and stimulates the migration of keratinocytes to the wound site and this promotes reepithelialization. The failure of the keratinocyte monolayer formation and delayed reepithelialization is characteristic of chronic wounds [Saarilho-Kere, 1994]. During wound healing keratinocytes undergo numerous gene expression changes and therefore are an appropriate cell type for the study of gene expression changes as well as cytokine and chemokine profiles that occur in response to *S. aureus* in both its planktonic and biofilm forms [Secor, 2012].

Chapter 2: Early effects of *Staphylococcus aureus* biofilm secreted products on inflammatory responses of human epithelial keratinocytes

Background

Each year millions of people are stricken with chronic wounds such as diabetic foot ulcers, pressure ulcers, and venous leg ulcers that contribute toward morbidity and mortality in the U.S. annually [Beckrich and Aronovitch, 1999; Boyko et al., 1996; Landi et al., 2007]. Recent evidence indicates that bacterial biofilms are present in chronic wounds more often than in acute wounds and are related to the wounds inability to heal [Gjodsbol et al., 2006; James et al., 2008; Serralta et al., 2001]. Bacterial biofilms consist of bacterial communities embedded in a self-made extracellular polysaccharide matrix that are often resistant to a variety of antibiotics [Stewart and Costerton, 2001]. Chronic wounds provide an excellent environment for this matrix due to the tissue surface for growth, poor blood flow, and poor oxygen flow that inhibit host cell defenses against the bacteria [Kirker et al., 2009]. The surface of chronic wounds allow bacteria to readily adhere to a surface and relinquish their planktonic state to form an aggregate of bacteria that enable the newly formed biofilms to better survive in their environment [Gotz, 2002]. The inhibitions of the host cell defenses allow the bacteria to cooperate in the distribution of nutrients, removal of wastes, and to resist host defense mechanisms [Serralta, 2001].

The establishment of *Staphylococcus aureus (S. aureus)* biofilms in chronic wounds relies on the bacterial community's ability to adhere to tissue. After attachment, the bacteria rapidly secrete cell signaling molecules that coordinate activities of the bacteria through a process known as quorum sensing [Fuqua et al., 1994]. Biofilm formation by *S. aureus* has been shown to be highly dependent on the staphylococcal accessory regulator (*sarA)* [Beenken et al., 2004] and to a certain degree on the (*icaABCD)* operon [Beenken et al., 2003; Crampro et al.1999; Deluarie et al., 2012; Valle et al., 2003;], and the *walRK* operon [Resch et al., 2005]. Mutations in either *sarA* or the *ica* operon have been associated with reduced capacity of *S. aureus* to form biofilms. There is significant evidence that the molecules in the matrix of biofilms lead to impermeability to antibiotics and host defenses [Thurlow et al., 2011]. While the idea that biofilms affect wound healing has been established, the exact role of biofilms in chronic wounds is not well understood [Stewart and Costerton, 2001]. Biofilm's resistance to antibiotics make the infections difficult

to resolve and more likely to become chronic or recurrent infections in part due to the inhibition of the re-epithelialization process [Schierle et al., 2009].

Wound healing is a complex series of pathogen and host cell interactions. Most chronic skin wound healing is mediated by keratinocytes [Loo and Halliwell, 2009; Singer and Clark, 1999]. The ability of keratinocytes to migrate to the site of the wound and respond to inflammatory signals to eliminate infection and complete the process of reepithelialization is important to the ability of chronic wounds to heal [Singer and Clark, 1999]. Keratinocytes have been shown to express toll-like receptors 1-6 and 9, which can allow them to act as a first responder against pathogenic microorganisms. For example, *S. aureus* can activate nuclear transcription factor kB (NF-κB) in keratinocytes. Activated NF-κB then translocate into the nucleus and induces the transcription of NF-κB controlled genes such as interleukin 8 (IL-8) and nitric oxide synthase (iNOS) [Mempel et al. 2003; Miller 2008]. The role that keratinocytes play in wound pathogenesis makes them an excellent model to investigate the pathogenesis of wound healing *in vitro*.

Inflammatory response of the host is important to the ability of wounds to heal. Biofilms have been shown to contribute to the failure of wounds to reepithelialize through the activation of the β-catenin/c-myc pathways which is in part attributed to the unresponsiveness of cells at the wounds edge [Cook et al. 2000; Hasan et al. 1997; Stojadinovic et al. 2005]. A part of that inflammatory response is the production of inflammatory cytokines that serve to mediate host immune responses. Recent evidence has revealed that *S. aureus* biofilms affect the gene regulation and cytokine production of keratinocytes and thus may affect the way that wounds heal [Secor et al., 2011]. The goal of this project was to further investigate and compare the effects of S. *aureus* biofilm secreted products and *S. aureus* planktonic secreted products on gene expression profiles and inflammatory responses of human epithelial keratinocytes (HEKa). The hypothesis is that products secreted by *S. aureus* growing as a biofilm actively impair wound healing by promoting inflammatory responses in keratinocytes. To test this hypothesis, we investigated and compared inflammatory responses of HEKa cells exposed to BCM and PCM at both transcriptional and translational level.

Methods

Preparation of biofilm conditioned media. An overnight culture of *S. aureus* ATCC 6538 in 5.0 ml of tryptic soy broth (TSB) was incubated statically at 37° C for 24 hours. Tissue culture inserts were placed in a 24 well plate and inoculated with 10 µl of overnight culture and 500 µl of TSB and inoculated at 37° C for 72 hours. Every 24 hours during that 72 hour period the TSB supernatant was removed, the inserts were moved to new wells in the 24 well plates, and 500 µl of fresh TSB was added to the wells. At the end of 72 hour period the TSB was removed and 500 µl of phosphate buffered saline pH 7.4 (PBS) was added and left for 1 hour to wash the remaining TSB from the tissue culture insert. After the removal of the PBS, 500 µl of Epilife media (Invitrogen, Carlsbad, CA) was added and incubated for 24 hours at 37° C. The new biofilm conditioned media was then removed from the well and filtered with a 0.45 µm syringe and collected in 15 ml centrifuge tubes. This BCM collecting and filtering procedure was repeated every 24 hours for 3 days. The collected BCM was then pooled and frozen at -20° C until it was needed.

Preparation of planktonic conditioned media. An overnight culture was created by inoculating a colony of *S. aureus* ATCC 6538 into 5.0 ml of TSB for 24 hours at 37° C on a rotary shaker set at 150 rpm. After incubation the *S. aureus* culture was centrifuged for 7 minutes at 1500 rpm. The supernatant was then replaced with PBS, and the pellet was re-suspended by thoroughly mixing with a pipette. The *S. aureus* was then centrifuged for 7 minutes at 1500 rpm and the PBS was decanted. Five milliliters of Epilife media with human keratinocyte growth supplement was then added to the washed *S. aureus* culture and mixed thoroughly with a pipette. The *S. aureus* in the Epilife media was then incubated for 24 hours at 37° C on a rotary shaker set at 150 rpm. After 24 hours the culture was centrifuged at 1500 rpm for 7 minutes and the supernatant was decanted and filtered with a 0.45 µm syringe and stored at -20° C until needed.

Culturing of human keratinocytes. HEKa cells (Invitrogen, Carlsbad, CA) were seeded into a T-25 and T-75 tissue culture flasks at 1 x 10^4 cells/ml along with Epilife media with human keratinocyte growth supplement and 100 µg/ml and 100 U/ml of pen/strep and incubated at 37° C in a CO_2 incubator. Every 48 hours the Epilife media was changed until the cells reached 80-90% confluence. In our hands, HEKa cells would start to become senescent at about passage 8 or 9. To prevent cell senescent from altering the results all experiments were performed with cells at passages 3 or 4.

XTT HEKa cell viability assay. BCM and PCM that was created previously were warmed to room temperature and diluted to1 mg/ml in Epilife media with human keratinocyte growth supplement. HEKa cells were cultured until they were at 80-90% confluence and then the original Epilife media with human keratinocyte growth supplement media was removed and 300 µl of either the PCM, BCM or media was added to the wells in triplicate and incubated for 0, 2, 4, 8, 24, and 48 hours. At the designated time 60 µl of activated 2, 3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) solution was added and absorbance read at 450 nm on a Thermo Scientific Multiskan MCC micro plate reader (Fisher Scientific, Pittsburgh PA).

Genomic responses, statistics and bioinformatics analyses. HEKa cell genomic responses to PCM, BCM and Epilife media were also evaluated. HEKa cells were cultured and plated in a 24 well plate as described above and were grown to 80-90% confluence. After reaching 80-90% confluence the cells were exposed to 1.0 ml of PCM, BCM or Epilife media for a period of 0 and 2 hours. At the designated times points the BCM, PCM, and Epilife media was removed, and the cells liberated from the wells for RNA extraction using Qiagen RNAeasy Cell Protect kit (Qiagen Inc. Valencia, CA). The concentrations of the RNA were determined using a Nanodrop spectrophotometer (Fisher Scientific, Pittsburgh PA). RNA was diluted to a 0.1 µg/ml concentration in RNase-free water and transported to Oklahoma Medical Research Foundation (OMRF) for microarray analysis. RNA quality was assessed using capillary gel electrophoresis (2100 Bioanalyzer, Agilent Technologies, Santa Clara, CA). Samples were labeled using Illumina TotalPrep RNA Amplification kit (Ambion/Life Technologies, Carlsbad, CA) and hybridized to whole genome gene expression microarrays (Illumina Human HT-12 v4 BeadChips, Illumina Inc., San Diego, CA) according to manufacturers' instructions. Microarrays were stained and washed under high stringency conditions and were then scanned on an Illumina iSCAN scanner. Signal intensity values were obtained using Genome Studio software (Illumina, v2011.1) and quantile normalized and log transformed using MatLab software (Mathworks, Inc., Natick MA) prior to importing into BRB-Array Tools (National Cancer Institute, Biometric Research Branch, Rockville, MD). Genes were then filtered using the Log Expression Variance Filter to screen out genes that are not likely to be informative based on the variance of each gene across the arrays. Biological replicates for each group were designated and statistically significant differentially expressed genes were identified using a 5% false-discovery rate and a minimum

1.5 fold change between the BCM and the PCM samples [Benjamin et al., 1995]. Bioinformatics analyses of differentially expressed genes were performed using Ingenuity Pathways Analysis software (Ingenuity Systems, Redwood City, CA).

Detection of inflammatory cytokines. HEKa cells were cultured to 80-90% confluence in 24 well plates 1.0 ml of BCM, PCM or Epilife media without pen/strep was added in triplicate cultures for periods of 0, 2, 4, and hours. At the designated time points the BCM, PCM and Epilife media was collected and stored in 1.5 ml microcentrifuge tubes at -20^{o} C until ELISA tests were performed. The concentrations of cytokines in the PCM, BCM, and Epilife media products that were exposed to HEKa cells were quantified and analyzed. Quantification of TNF-α IL-1, IL-6, and IL-8 was performed using the corresponding Quantikine Colorimetric sandwich ELISA assay kit (R&D Systems, Minneapolis, MN) following the manufacturer's instructions. CXCL2 was quantified using Abnova Enzyme-Linked Immunoabsorbant Sandwich Elisa Assay Kit (Abnova Industries, Taipei City, Taiwan).

Nitric oxide detection. Cell culture supernatants were collected, and the accumulation of nitrite (NO_2^-), a stable end product of NO formation, was measured as an indicator of NO production. The concentration of nitrite in the samples was calculated from a standard curve of sodium nitrite [Granger et al., 1991]. Briefly, at four and eight hours post exposure one hundred microliters of supernatants were collected from HEKa cells exposed to PCM, BCM, or control media samples and added in triplicate to the 96 well plates. One hundred microliters of Griess reagent was then added to all wells and incubated at room temperature for 15 minutes, and then absorbance was measured at 570 nm. After incubation the optical densities of each well were read on a Thermo Scientific Multiskan MCC micro plate reader (Fisher Scientific, Pittsburgh PA) at 570 nm.

Statistical analysis. To determine if there were significant differences in the cell viability of HEKa cells exposed to PCM, BCM and the control media two-tailed t-tests were completed. To determine if there were significant differences in the mean concentration of the cytokines or nitric oxide between three types of materials, two-tailed t-tests were completed along with ANOVA and Tukey's statistical methods. An ANOVA resulting in a significant p-value ($p < 0.05$) was followed by Tukey's Honestly Significant Difference to determine specifically which means were significantly different.

Results

HEKa cell viability. HEKa cell viability experiments were conducted to determine at which point in time if any products secreted by *S. aureus* growing as a biofilm or *S. aureus* growing planktonically significantly reduced viability of HEKa cells. The XTT viability assay was used to determine the loss of HEKa viability after exposure to PCM, BCM, or Epilife media. After 8 hours exposure there was a significant reduction of keratinocyte viability in PCM and BCM exposed cells (Figure 1). Based on these results it was established that sampling time points of 2 hours for microarray analysis and 4 and 8 hours for inflammation analysis would be used.

Transcriptional responses of HEKa cells exposed to BCM or PCM. We predicted that HEKa cells exposed to *S. aureus* biofilm secreted products would display differential genes expression when compared to HEKa cells exposed to *S. aureus* planktonically secreted products or the media controls. To test this hypothesis we extracted HEKa cellular RNA after 2 hours of exposure to BCM, PCM, or media controls and subjected the RNA to Illumina microarray analysis in order to evaluate differential gene expression. The data was filtered so that only genes that had a 1.5 fold changes or greater over the media control were shown (Table 1). Analysis revealed that HEKa cells that were exposed to BCM as well HEKa cells exposed to PCM had genes associated with inflammation, apoptosis, and nitric oxide production were upregulated when compared to the media controls. When cells exposed to BCM were compared to those cells exposed to PCM, the cells exposed to BCM had an increase in the number and fold increase of transcriptional products. The microarray analysis identified 43 genes that were uniquely expressed in BCM exposed HEKa cells with a 1.5 or greater fold change in expression that were not expressed in HEKa cells exposed to PCM or the media control (Table 1). Of these genes 42 had an upregulation of expression over the control. Of these upregulated genes, 19 are associated with inflammatory response in eukaryotic cells. Eleven genes associated with inflammation were also differentially expressed in HEKa cells exposed to PCM, 9 of which were also differentially expressed in cells exposed to BCM (Table 2) and (Figure 2A). The largest increase in gene expression in HEKa cells exposed to BCM for genes associated with

inflammatory response versus HEKa cells exposed to BCM were DUSP1, CXCL2, IL-8, ATF3, IL-6 and NFKBIA (Table 1).

Nitric oxide (NO) is an important molecule for inflammation and the reepithelialization of skin. Low concentrations of NO have been found to inhibit adhesion molecule suppression cytokines and chemokine synthesis and leukocyte adhesion and migration [Luo and Chen 2005, Rizk et al., 2004]. This promotes collagen production either directly or through mediators and thus affects collagen synthesis or breakdown in the wound [Schaffer et al., 1996]. While low amounts of NO can be beneficial to wound repair large amounts of NO generation has been shown to be toxic, pro-inflammatory, and cause the wound to enter a cycle where healing does not occur [Frank et al., 2002; Jones et al., 2010]. Because of this, microarray results were filtered to look for genes that are associated with the production of nitric oxide (NO). The results showed that at two hours after treatment there were several differentially expressed genes associated with NO production that were uniquely upregulated in HEKa cells exposed to BCM (Table 1). This upregulation was not seen in HEKa cells treated with PCM for two hours. Of these genes DUSP1, JUN, IL-6, and ADM were up regulated the most. Most of the genes above are linked to the production of IFN-γ and TNF-α. Both IFN-γ and TNF-α have been linked to an increase of NO in human keratinocytes [Hick et al., 1992]. Oxidative stress has been found to increase the amount of IFN-γ produced by the cells which in turn regulates the transcription of DUSP1 and ADM which ultimately leads to increases in apoptosis. IFN-γ along with TNF-α stimulates the production of nitric oxide in keratinocytes as a response to oxidative stress [Soneja et al., 2005]. IFN-γ has been found to increase the production of NO in keratinocytes as much as 20-30%. IFN-γ has also been linked to an increase in production of IL-6 along with NO production [Hashiola et al., 2007]. In addition to upregulation of genes associated with NO production, we observed down-regulation of ARG1, which is known to be associated with NO production in both BCM and PCM, exposed HEKa cells relative to control media (Table 3) and (Figure 2B). Overproduction of ARG1 has been linked to keratinocytes overproducing NO in patients with psoriasis and basal carcinomas. The inhibition of ARG1 may then allow NO to inhibit cell proliferation and the failure of wounds to reepithelialize [Bruch-Gerharz et al., 2003].

Inflammatory cytokine responses in HEKa cells exposed to BCM or PCM. In an effort to corroborate the microarray results, we assessed the production of the protein levels of several of the most markedly upregulated genes associated with an inflammatory response. ELISAs were used to measure the production of IL-1, IL-6, IL-8, TNF-α, and CXCL2. Cytokine measurements were performed at 4 and 8 hours post exposure to BCM or PCM. ELISA data showed that as soon as 4 hours post exposure, there were significantly greater ($p < 0.05$) levels of IL-6, TNF-α, and CXCL2 in HEKa cells exposed to BCM compared to HEKa cells exposed to PCM or control media (Figure 3A). The levels of IL-6 and CXCL2 were even greater at 8 hours post exposure and the significant differences between the groups were maintained (Figure 3B). In addition to these three cytokines, the levels of IL-8 were also significantly greater ($p < 0.05$) after 8 hours of exposure in HEKa cells exposed to BCM compared to HEKa cells exposed to PCM, or controls (Figure 3B). PCM exposed HEKa cells did not produce significantly greater cytokine levels compared to the media controls at either time point.

NO responses in HEKa cells exposed to BCM or PCM. The microarray data revealed an increase in the expression of genes associated with the production of nitrite in BCM-exposed HEKa cells. In an effort to corroborate these results, we performed nitric oxide assays on the HEKa cell culture supernatants to quantify the amount of nitrate produced by the HEKa cells exposed to PCM, BCM and control media. The nitrite assay data showed that HEKa cells exposed to BCM produced significantly greater ($p < 0.05$) amounts of nitrite at both 4 and 8 hours post exposure than HEKa cells exposed to PCM or the media controls (Figure 4). There were no detectable levels of nitrite from the media controls.

Discussion

The data collected in this study reveal a clearer picture of the role that *S. aureus* biofilms play on cultured keratinocytes. Keratinocytes serve as the primary cell type in the epidermis and primarily function in providing a barrier between the external and internal environment. When breaks in this barrier occur, basal keratinocytes migrate to the site and reepithelialization ensues. Chronic wounds are characterized by prolonged inflammation and the failure of wound reepithelization [Kirker et al., 2009]. Chronic wounds activate a number of inflammatory pathways that lead to the prevention of keratinocyte migration, growth, and differentiation and thus failure of wounds to heal [Stojadinovic et al., 2005]. Due to their importance

in chronic wound pathogenesis HEKa cells were chosen for experiments on the effect of biofilms on early chronic wound pathogenesis. Kirker et al. [2009] demonstrated that the viability of HEKa cells was significantly reduced when exposed to BCM or PCM for 24 hours. Results from our HEKa cell viability assays showed that viability was significantly reduced by BCM or PCM in as little as 8 hours of exposure (Figure 1), indicating that our findings are in general agreement with previous findings of Kirker et al.[Kirker et al., 2008]. Visual inspection of the cells showed morphological differences between HEKa cells exposed to the two treatment conditions. Evidence of cellular stress in the form of rounding of cell membranes and decreased culture confluence were observed in HEKa cells exposed to BCM after 8 hours of exposure that was not seen in HEKa cells exposed to PCM or the control media (data not shown). Based on the viability assay results and these morphological changes, transcriptional changes were measured at two hours after treatment to detect early transcriptional changes in cell populations with high viabilities. For inflammatory cytokine response and NO production 4 and 8 hour time intervals were used. These time points were selected in an effort to measure downstream effects of transcription and inflammatory responses.

Increased apoptotic effects in HEKa cells exposed to *S. aureus* biofilm secreted products provides greater understanding of the pathogenesis of wound healing. Decreased cell viability and the inability of wounds to heal have been linked to chronic wounds associated with biofilms [Kirker et al., 2008]. Figure 2C shows alterations in transcription of genes associated with apoptosis in BCM- and PCM-exposed HEKa cells. This correlates with our cell viability data (Figure 1) which reveals a statistically significant loss of HEKa cell viability in BCM and PCM after 8 hours. There are several definitions of what an actual chronic wound is, but some common themes are a prolonged inflammatory phase to the wounds and the failure of the wound to respond to standard treatments [Fletcher, 2008]. These wounds fail to reepithelialize which is due in part to the decrease of cell production and the increase in cell death. Our data indicate that there is an increase in apoptotic effect in HEKa cells exposed to BCM over PCM or the control conditions.

Microarray analysis was performed on RNA gathered after 2 hours exposed to BCM, PCM, or the media control the samples of HEKa cells exposed for 0 hours were used as a control. The data were then filtered in order to identify differentially expressed genes that were associated with inflammatory

responses, apoptosis and nitric oxide production in HEKa cells. There was an increase in transcriptional activity in HEKa cells exposed to BCM for genes associated with inflammation, apoptosis and NO production (Figure 2). In HEKa cells exposed to BCM some of the largest upregulation of transcriptional activity came from the genes CXCL2, IL-8, DUSP1, and ATF3. The DUSP1 gene is important for the regulation of p38 activation of LPS-activated macrophages. The p38 pathway plays a central role in multiple pathways associated with inflammatory response in many cell types [Cuenda and Rousseau 2007; Schieven 2009]. The p38 MAPK pathway has been found to play a role in the production of inflammatory cytokines namely IL-1 and TNF-α but has also been found to contribute to the production of IL-8 in response to IL-1 osmotic shock and IL-6 in response to the production of TNF-α [Newton and Dixit, 2012]. NFKBIA like DUSP1 is an important transcriptional regulator that induces innate and adaptive immune responses. NFKBIA is one of the genes that assist in the regulation of the magnitude and duration of inflammatory responses. One of its key roles is to prevent the inflammatory response from destroying excessive amounts of tissue [Newton and Dixit, 2012]. Some of the other genes up regulated are associated with the production of inflammatory cytokines or chemokines. CXCL2, IL-6 and IL-8 are well known cytokines or chemokines which play important roles in mediating inflammatory responses to pathogen. CXCL2 and IL-8 are pro-inflammatory chemokines that assist in the mediation of neutrophil migration as well as the migration of other cellular and humeral factor components to the site of an infection. TNF-α, IL-6 and IL-8 were also upregulated but the fold change in expression over the control was much lower and the increase in expression was only seen in HEKa cells exposed to BCM. TNF-α, IL-6 and IL-1 are all multifunctional cytokines with a wide variety of functions. These three cytokines are interrelated with IL-1 and TNF-α inducing IL-6 and IL-6 in turn playing a role in the regulation of TNF-α [Abraham et al., 2000]. TNF-α plays a role in tissue repair, inflammation and regulation of apoptosis as well as the activation of transcriptional factors such as NFKB that are important to several processes including growth, death, and inflammation and stress responses [Banno et al., 2004]. While NF-κB is not regulated at the RNA level and is not be expected to be differentially expressed, Ingenuity Pathways Analysis software predicted its increased activation based on the expression patterns of 28 genes in the microarray results from BCM-treated but not PCM-treated HEKa cells relative to media controls ($p = 1.06 \times 10^{-11}$). DUSP1 is produced in human skin cells and specifies a protein with structural features similar to members of non-receptor-type

protein-tyrosine phosphatase family which inactivates MAPKs. MAPKs play an important role in the human cellular response to environmental stress as well in the negative regulation of cellular proliferation [Lie et al., 2008]. This can lead to an increase in cell death and thus contribute to *S. aureus* biofilms' negative effects on the ability of HEKa cells ability to respond to bacterial challenges and prevent apoptosis and promote reepithelialization.

Seven cytokines or chemokines commonly produced by keratinocytes in response to pathogens were tested using sandwich ELISAs [Abraham et al., 2000; Fahay et al., 1990; Trengove et al., 2000]. After 4 hours, cells exposed to BCM showed statistically significant increases in concentrations of IL-6, TNF-α, and CXCL2 in HEKa cells exposed to BCM over HEKa cells exposed to PCM and the control media. The increase in inflammatory cytokine and chemokine responses after 4 hours of exposure to PCM, BCM, and the media control were in general agreement with earlier research by Secor et al. [2001] who showed increases in inflammatory responses by HaCaT cells exposed to *S. aureus* biofilm products. HEKa cells exposed to PCM, BCM and the control media were also tested by ELISA's for the same seven cytokines and chemokines. After 8 hours of exposure, levels of IL-8, along with previously elevated IL-6, TNF- α, and CXCL2 significantly increased in HEKa cells exposed to BCM over HEKa cells exposed to PCM and the media control. At the 8 hour time point HEKa cells treated with BCM showed a statistically significant increase in cytokines versus HEKa cells treated with PCM. These findings support the current hypothesis that biofilm-secreted products differ and have more dramatic effects than secreted products from planktonically grown bacteria which may contribute to difference between chronic and acute wounds [James et al., 2008]. The creation of biofilms enables bacteria to increase their interaction with one another and thus resist antibacterial and environmental pressures [Serralta et al., 2001]. These biofilms are not only a congregate of bacterial cells but are also held together by a polysaccharide matrix. Understanding the components of the exopolysaccharide matrix produced by bacterial biofilms along with the difference in morphology of the cells may provide clues to the mechanisms of biofilms and why they produce the differential inflammatory responses that we have observed.

An unexpected finding from the microarray analysis was the upregulation of genes associated with nitric oxide production in HEKa cells exposed to BCM. Nine genes were up regulated in HEKa that were

not found in control samples. Eight of those genes were unique to HEKa cells exposed to BCM and were not found in HEKa cells exposed to planktonically secreted products. Despite the genes associated with nitric oxide production there was not a significant upregulation of iNOS or NOS2 which are most often associated with nitrite production in cells. The lack of significant iNOS an NOS2 upregulation may have been due to the short time between the exposure of the HEKa cells to the PCM and BCM and the time of mRNA collections. Schnorr et al.[2003] reported that iNOS expression in keratinocytes was not detected until 4-8 hours after exposure to inflammatory cytokines and maximal expression does not occur until 24 hours post exposure [Schnorr et al., 2003]. Nitric oxide plays a key role in acute wound repair. Nitric oxide synthesis increases wound healing by enhancing collagen deposition within the wound and in dermal fibroblasts to enhance the mechanical strength of the tissue [Granger et al., 1991; Rizk et al., 2004]. However, high concentrations NO may actually inhibit healing [Schaffer et al., 1996]. In this study, we observed upregulation of genes associated with NO production in cells treated with BCM, and tested the downstream effects of such genes by performing nitrite assays on culture supernatants. These nitrite assays were done in order to measure nitrite as an index of NO formation. Statistically significant increases in the amount of nitrite produced by HEKa cells exposed to BCM compared to HEKa cells exposed to PCM or just Epilife media were detected. These results add to the understanding of NO role in chronic wounds as opposed to acute wounds. The role of NO in biofilm production and dispersal is controversial. Falsetta et al. [2010] found that in terms of *Neisseria gonorrhea* biofilms there was a high level of NO which seemed to aid the biofilm's growth. Other studies have found that NO has been linked to the dispersal of *Pseudomonas aeruginosa* biofilms as well the prevention of biofilm formation in *S.aureus* and *Escherichia coli* [Barraud et al., 2009; Cai et al., 2012]. It has been suggested that the disparate results are due to concentration of NO that is induced in the system. NO in large concentrations can be toxic to eukaryotic cells and in fact eukaryotic cells have developed defenses like metallothionein in response to oxidative stress [Frank et al., 2002]. Our results show that NO production by HEKa cells increased over time (Figure 4) as inflammatory effects of biofilm secreted products increased (Figure 3). Overproduction of reactive nitrogen species can impair cellular migration, proliferation, and synthesis of extracellular matrix that is important to wound healing by keratinocytes [Soneja et al., 2005]. This lack of agreement between studies

coupled with our results makes NO and its role in biofilm formation, dispersal, and/or chronic wound pathogenesis a topic that requires further investigation.

The majority of studies on immune responses to bacteria have been carried out with planktonically growing bacteria or cell components from planktonically growing bacteria. Infections due to *S. aureus* are characterized by strong inflammatory responses [Fournier et al., 2005; Nakane et al., 1995]. Several molecules such as Panton-Valentine leukocidin [Konig et al., 1995] sphingomyelinase [Waley et al., 1996], peptidoglycan, lipoteichoic acid [Wang et al., 2000; Wang et al., 2004], superantigens [Dauwalder et al., 2006] as well as others have been shown to play a role in the inflammatory response directed towards *S. aureus*. It is possible that some of these same molecules may be responsible for the differences in inflammatory and nitric oxide responses we have measured; however, these molecules have not been specifically linked to *S. aureus* biofilms and chronic wound inflammation.

Conclusion

Studies presented in this investigation further our understanding of the role that *S. aureus* biofilms may play in chronic wound pathogenesis. We found greater transcriptional activity in HEKa cells exposed to BCM compared to HEKa cells exposed to PCM or the media controls. The increased transcriptional activity was corroborated by increases in inflammatory cytokine production, nitric oxide production, and reduced cell viability. The increase in nitrite production in HEKa cells exposed to BCM is an intriguing finding that could be an important biofilm-mediated factor that contributes to the failure of chronic wounds to heal and warrants further investigation. Experiments to further understand the role of nitric oxide in chronic wounds and studies to identify *S. aureus* biofilm secreted factors responsible for the increased inflammatory responses are planned.

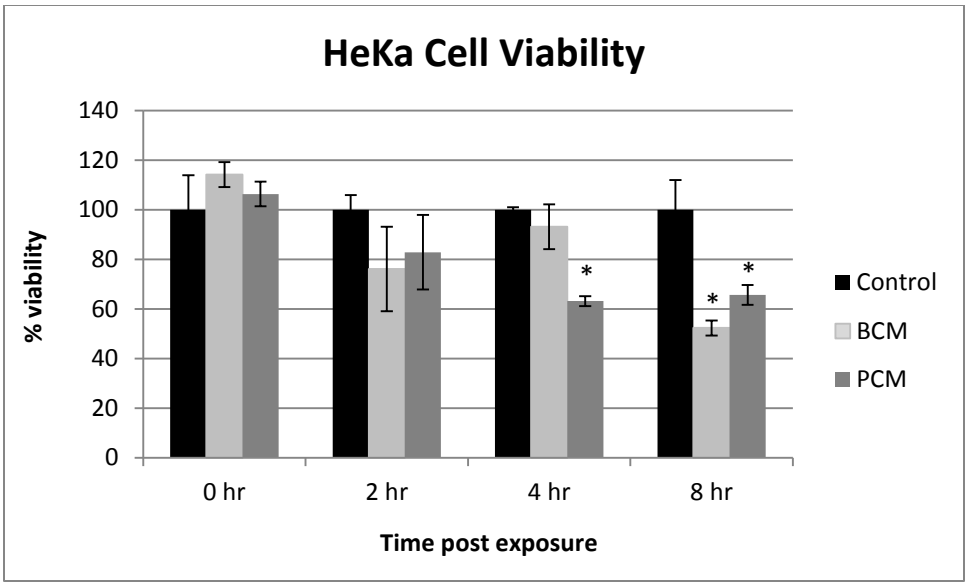

Figure 1. HEKa Cell Viability

HEKa cells were treated with Media, PCM or BCM for up to 8 hours. At 0, 2, 4, and 8 hours post exposure XTT was added and the absorbance read at 540 nm. Results represent the mean and standard deviation of three independent experiments. Percent viabilities are stated as percent viable of untreated controls at each time point.

(*) indicates a statistically significant difference between the treatment and the control ($p < 0.05$).

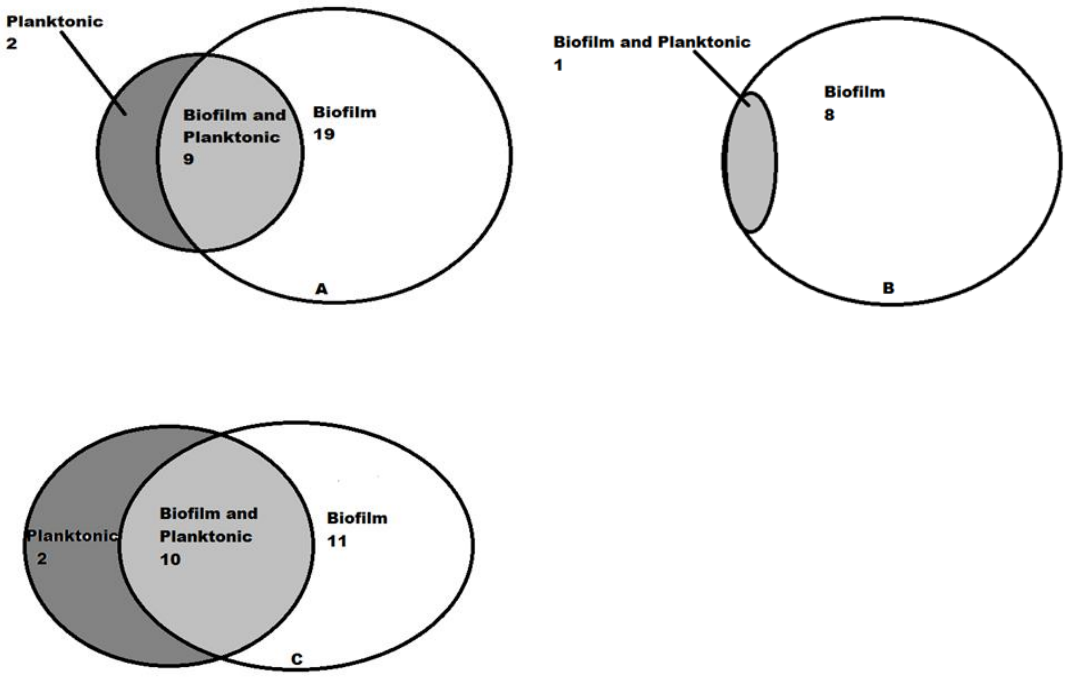

Figure 2. Venn diagrams representing the number of genes that were altered in HEKa cells exposed to BCM (on the right), PCM (on the left), or shared genes (in the middle).

The Venn diagram (A) represents genes associated with inflammation, (B) represents genes associated with NO production, and (C) represents genes associated with apoptosis.

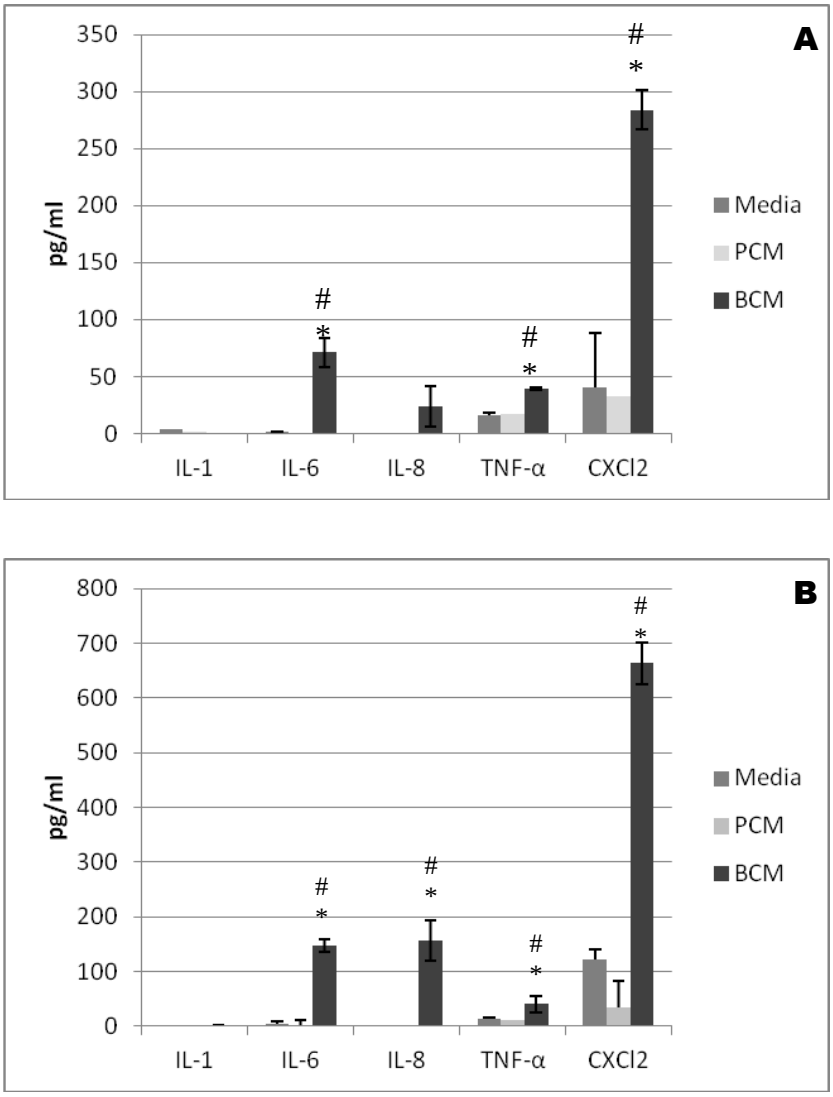

Figure 3. ELISA results for PCM, BCM, and control HEKa cells.

HEKa cells were treated with Media, PCM or BCM for up to 8 hours. The levels of cytokine production were measured after 4 and 8 hours of exposure. (A) Cytokine measurements after 4 hours of exposure. (B) Cytokine measurement after 8 hours of exposure. Measurements are reported in pg/ml concentrations. Analysis of variance (ANOVA) and Tukey's Honestly Significant Difference were performed to identify statistically significant differences. Results represent the mean and standard deviation of three independent experiments. (#) indicates a significant difference between PCM and BCM and (*) indicates a statistically significant difference between BCM and media ($p < 0.05$).

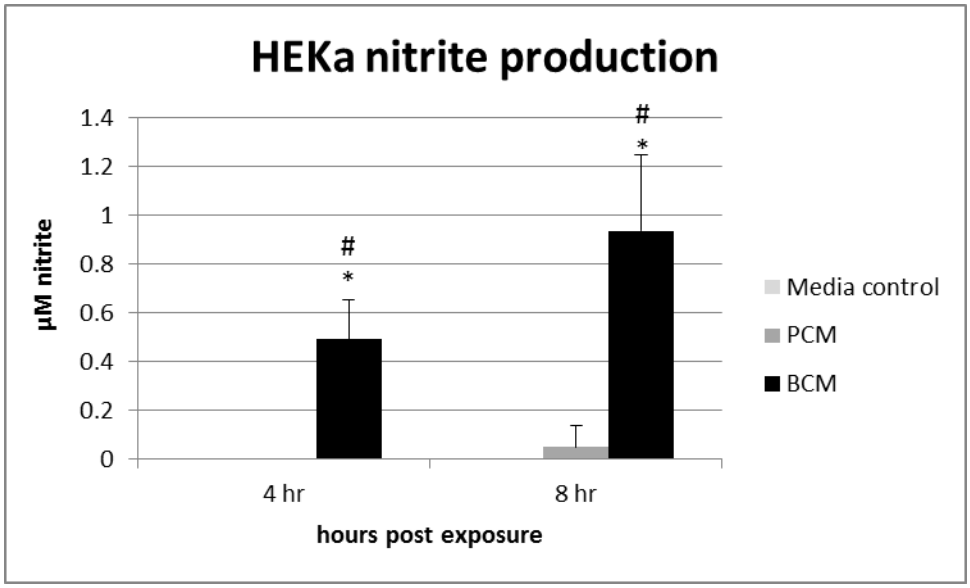

Figure 4. Nitrite Assay

HEKa cell culture supernatants were collected and treated with Griess Reagent and absorbance was measured at 570 nm. Measurements were taken at 4 and 8 hours. Measurements are reported in µM. ANOVA and Tukey's Honestly Significant Difference were performed to identify statistically significant differences. Results represent the mean and standard deviation of three independent experiments. (#) indicates a statistically significant difference between the PCM and BCM and (*) indicates a statistically significant difference between media and BCM ($p < 0.05$).

Table 1. Change in gene expression profiles for genes that were uniquely expressed in BCM exposed HEKa cells after 2 hours of exposure. Genes identified through Illumina microarray results filtered to represent a 1.5 or greater fold change over the expression in the control sample. "-"indicates less than a 1.5 fold change in expression over the control sample.

Gene Symbol (function)	Fold-change BCM v Control	Fold-change PCM v Control
CXCL2 (inflammation)	15.082	-
IL8 (inflammation)	9.530	-
DUSP1 (inflammation/NO prod.)	9.061	-
IL6 (inflammation/NO prod.)	4.623	-
NFKBIA (inflammation)	4.093	-
EFNA1 (inflammation)	3.990	-
TNFAIP3 (inflammation)	3.964	-
ADM (inflammation/NO prod.)	2.917	-
CXCL1 (inflammation)	2.850	-
IL1B (inflammation/NO prod.)	2.586	-
ZFP36 (inflammation)	2.540	-
IFI27 (inflammation)	2.411	-
TNF (inflammation/NO prod.)	2.333	-
IL1A (inflammation/NO prod.)	2.113	-
PTGS2 (inflammation/NO prod.)	2.084	-
SMAD7 (inflammation)	2.073	-
IL20 (inflammation)	1.655	-
IL24 (inflammation)	1.540	-
MMP1 (inflammation)	-1.650	-
FOS (apoptosis)	8.433	-
ZC3H12A (apoptosis)	4.361	-
NR4A2 (apoptosis)	4.121	-
SGK1 (apoptosis)	3.045	-
CYP1B1 (apoptosis)	2.709	-
CYR61 (apoptosis)	2.587	-
BMP2 (apoptosis)	2.481	-
SLC25A24 (apoptosis)	2.467	-
CEBPD (apoptosis)	2.419	-
IFI27 (apoptosis)	2.411	-
BIRC3 (apoptosis)	1.643	-
HES1 (growth & proliferation)	4.280	-
DUSP10_v3 (growth & proliferation)	4.091	-
EFNA1 (growth & proliferation)	3.990	-
PPP1R15A (growth & proliferation)	3.582	-
JUN (growth & proliferation/NO prod.)	3.523	-
PPP1R10 (growth & proliferation)	3.099	-
HBEGF (growth & proliferation)	3.053	-
CYR61 (growth & proliferation)	2.587	-
CEBPD (growth & proliferation)	2.419	-
JUNB (growth & proliferation)	2.405	-
SMAD7 (growth & proliferation)	2.073	-
DUSP10_v1 (growth & proliferation)	1.764	-
CYR61 (wound healing)	2.587	-

Table 2. Change in gene expression profiles for genes associated with Inflammation. Genes identified through Illumina microarray results filtered to represent a 1.5 or greater fold change over the expression in the control sample. "-"indicates less than a 1.5 fold change in expression over the control sample.

Gene Symbol	**Fold-change BCM v Control**	**Fold-change PCM v Control**
ATF3 (inflammation)	8.342	1.663
FOXA2 (inflammation)	3.139	2.715
BCL6 (inflammation)	2.801	1.515
SERPINB13 (inflammation	2.513	2.768
RHOB (inflammation)	2.443	1.464
MMP28 (inflammation)	2.117	1.672
IL1F9 (inflammation)	1.662	2.438
PBK (inflammation)	-2.057	-1.882
TK1 (inflammation)	-2.168	-2.029
CXCL2 (inflammation)	15.082	-
IL8 (inflammation)	9.530	-
DUSP1 (inflammation/NO prod)	9.061	-
IL6 (inflammation)	4.623	-
NFKBIA (inflammation)	4.093	-
EFNA1 (inflammation)	3.990	-
TNFAIP3 (inflammation)	3.964	-
ADM (inflammation)	2.917	-
CXCL1 (inflammation)	2.850	-
IL1B (inflammation/ NO prod)	2.586	-
ZFP36 (inflammation)	2.540	-
IFI27 (inflammation)	2.411	-
TNF Inflammation)	2.333	-
IL1A (inflammation/ NO prod)	2.113	-
PTGS2 Inflammation/NO prod)	2.084	-
SMAD7 (inflammation)	2.073	-
IL20 (inflammation)	1.655	-
IL24 (inflammation)	1.540	-
MMP1 (inflammation)	-1.650	-
IL28RA (inflammation)	-	1.589
CXCR7 (inflammation)	-	2.435

Table 3. Gene expression profiles for genes associated with NO production. Genes identified through Illumina microarray results filtered to represent a 1.5 or greater fold change over the expression in the control sample. "-"indicates less than a 1.5 fold change in expression over the control sample.

Gene Symbol	Fold-change BCM v Control	Fold-change PCM v Control
ARG1 (NO prod)	-3.042	-2.499
DUSP1 (NO prod)	9.061	-
IL6 (NO prod)	4.623	-
JUN (NO prod)	3.523	-
ADM (NO prod)	2.917	-
IL1B (NO prod)	2.586	-
TNF (NO prod)	2.333	-
IL1A (NO prod)	2.113	-
PTGS2 (NO prod)	2.084	-

Chapter 3: Summary and Conclusions

In an effort to better understand the effect of *S. aureus* biofilms on human keratinocytes, we treated human epithelial keratinocytes with secreted products from *S. aureus* bacteria grown planktonically and as a biofilm. The secreted producets of *S. aureus* cell grown planktonically and as a biofilm were introduced to HEKa cells and data was collected to determine if there was a differential inflammatory response. We hypothesized that S. *aureus* growing as biofilms actively promote inflammatory responses in human epithelial keratinocytes. To test this hypothesis we performed a series of experiments to better understand differential response to *S. aureus* bacterial biofilms.

To test inflammatory responses we evaluated differential gene expression in HEKa cells treated with the BCM, PCM and control conditions. Transcriptional profiling was done using illumine microarray technology. The data from the microarray analysis was filtered to identify genes associated with inflammation, apoptosis, and nitric oxide production. In the case of inflammation, apoptosis and nitric oxide produced the HEKa cells treated with BCM showed an increase of genes transcribed. To confirm the increased inflammatory response seen in the microarray data we performed an ELISAs on five different cytokines and chemokines. We chose to test IL-1, IL-6, IL-8,TNF-α, and CXCL2 which are cytokines that are commonly produced by keratinocytes in response to infection [Secor et al., 2011]. ELISA assays were done on HEKa cells at four and eight hours after treatment with BCM, PCM, or control conditions. After four hours there was a significant difference in the production of IL-6, TNF-α and CXCL2 between HEKa cells treated with *S. aureus* cells grown in biofilms and cells grown planktonically and under control conditions. After eight hours, in addition to IL-6, TNF-α, and CXCL2, IL-8 production was also significantly elevated in HEKa cells exposed to BCM compared to HEKa cells exposed to PCM or control conditions. These data confirmed that there was a differential inflammatory response. Further studies are wanted to determine what secreted components or combination of secreted components from *S. aureus* biofilms cause the increased inflammatory response.

One of the more surprising findings in this survey was the upregulation of genes associated with nitric oxide production. Confirmation of nitric oxide production was done using a nitrite assay. The results

confirmed that there was a significant increase in the production of nitric oxide in HEKa cells exposed to BCM when compared to nitric oxide levels of HEKa cells exposed to PCM and control conditions.

As a part of the normal inflammatory process some loss of cell viability is expected. In this study microarray analysis showed that genes associated with apoptosis were more highly expressed in HEKa cells treated with BCM than HEKa cells treated with PCM or control conditions. Cell viability assays also showed that there was a greater loss of cell viability in HEKa cells treated with BCM. Furthur studies are needed to determine if the loss of cell viability is due to apoptosis or necrosis and possibly how that relates to cytokine or NO production.

The results of all of these tests indicate that products secreted by *S. aureus* growing in biofilms actively promote inflammatory responses in human keratinocytes, which may play a direct role in the prolonged inflammation and lack of re-epithelialization of chronic wounds. Work will now focus on identifying the products in *S. aureus* biofilm secretions that promote chronic inflammation and lack of re-epithelialization, as well as understanding the role of nitric oxide in chronic wound pathogenesis. Future work involving mouse wound models may be valuable in determining if the supernatants can inhibit healing in a real wound. This study looked at one particular strain of *S. aureus* but future studies should also evaluate the effects of supernatants from different strains of *S. aureus* to determine if there are different inflammatory effects. The use of different strains of the bacterium may determine if the inflammatory promoting factors are strain specific or highly conserved among strains. The fundamentally new knowledge gained through this work is expected to foster the development of more effective treatments, which will promote the healing process and ultimately reduce the amount of morbidity, mortality and medical response associated with chronic wounds.

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