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Loss of viability and induction of apoptosis in human keratinocytes exposed to *Staphylococcus aureus* biofilms in vitro

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ABSTRACT

Bacteria colonizing chronic wounds are believed to exist as polymicrobial, biofilm communities; however, there are few studies demonstrating the role of biofilms in chronic wound pathogenesis. This study establishes a novel method for studying the effect of biofilms on the cell types involved in wound healing. Cocultures of *Staphylococcus aureus* biofilms and human keratinocytes (HK) were created by initially growing *S. aureus* biofilms on tissue culture inserts then transferring the inserts to existing HK cultures. Biofilm-conditioned medium (BCM) was prepared by culturing the insert-supported biofilm in cell culture medium. As a control planktonic-conditioned medium (PCM) was also prepared. Biofilm, BCM, and PCM were used in migration, cell viability, and apoptosis assays. Changes in HK morphology were followed by brightfield and confocal microscopy. After only 3 hours exposure to BCM, but not PCM, HK formed dendrite-like extensions and displayed reduced viability. After 9 hours, there was an increase in apoptosis ($p \leq 0.0004$). At 24 hours, biofilm-, BCM-, and PCM-exposed HK all exhibited reduced scratch closure ($p \leq 0.0001$). The results demonstrated that soluble products of both *S. aureus* planktonic cells and biofilms inhibit scratch closure. Furthermore, *S. aureus* biofilms significantly reduced HK viability and significantly increased HK apoptosis compared with planktonic *S. aureus*.

Keratinocytes are the major cell type of the epidermis, which serves as the primary barrier between the external environment and the internal tissues. In this capacity, the epidermis also functions as a barricade to microorganisms, toxins, and various antigens. When the barrier is breached due to wounding, basal keratinocytes from the wound edges or dermal appendages migrate over the open wound to reestablish the barricade in a process called reepithelialization. Chronic wounds, such as diabetic foot ulcers, venous leg ulcers, and pressure ulcers, are characterized by prolonged inflammation, an altered wound matrix, and the failure to reepithelialize.

Chronic wounds are also characterized as supporting a diverse microbial flora. A literature review by Bowler examined culture data from 62 published studies dating between 1969 and 1997.[1] The most predominant wound isolate in both chronic and acute wounds was *Staphylococcus aureus* (reported in 63% of the studies), followed by coliforms (45%), *Bacteroides* spp. (39%), *Peptostreptococcus* spp. (36%), *Pseudomonas aeruginosa* (29%), *Enterococcus* spp. (26%), and *Streptococcus pyogenes* (13%).[1] Using molecular techniques, Dowd et al.[2] also demonstrated vast bacterial diversity within chronic wounds. The most prevalent species included *Staphylococcus*, *Pseudomonas*, *Peptoniphilus*, *Enterobacter*, *Stenotrophomonas*, *Finegoldia*, and *Serratia* spp.

It has been speculated that bacteria colonizing chronic wounds exist as a biofilm.[3–7] Biofilms represent bacterial communities surrounded by extracellular polysaccharide matrix. Such communities are often polymicrobial and resistant to antimicrobials. Chronic wounds are an ideal environment for bacterial infection and biofilm formation. The wound remains open for a prolonged period of time, increasing the odds of bacterial infection. The wound bed provides a surface for growth, and poor blood flow and hypoxia discourage native defenses.[8] Studies have shown that wounds inoculated with bacteria form biofilms.[6,9] Furthermore, in a recent study by James et al.,[10] 60% of chronic wound specimens were characterized as containing biofilm compared with 6% of acute wound specimens. Despite the prevalence of biofilms in wounds, there are few data illustrating the role of biofilms in chronic wound pathogenesis. This study establishes a novel method for directly studying the effect of biofilms on the cell types involved in wound healing. Specifically, it examines the effect of *S. aureus* biofilms on keratinocyte morphology, viability, and scratch closure.

METHOD AND MATERIALS

Cell culture

Normal human keratinocytes (HK) were isolated from newborn foreskin using methods previously described[11] and in accordance with the University of Washington

Institutional Review Board. Cells were maintained in EpiLife® keratinocyte growth medium (Cascade Biologics, Portland, OR) supplemented with human keratinocyte growth supplement (HKGS; Cascade Biologics) and penicillin and streptomycin (P/S), 100 U/mL and 100 μg/mL, respectively (Hyclone, Logan, UT). All cultures were kept in a humidified 5% CO_2 incubator at 37 °C. Experiments were conducted with cell passages 4–10, using EpiLife® medium supplemented with HKGS and without P/S, unless noted otherwise.

Biofilm growth and biofilm-conditioned medium (BCM)

A clinical isolate of *S. aureus* was isolated from a chronic wound using methods previously described[10] and in accordance with a protocol approved by the Montana State University Institutional Review Board. *S. aureus* biofilms were grown on tissue culture inserts, using techniques similar to the colony biofilm method described by Anderl et al.[12] Briefly, an overnight culture of *S. aureus* was diluted in tryptic soy broth (TSB) to an optical density of 0.05 at 600 nm. Tissue culture inserts (10 mm diameter, pore size 0.2 μm; Nalge Nunc International, Rochester, NY) were inoculated with 10 μL of the diluted culture. The inserts were then placed in a 24-well plate, each well containing 200 μL TSB, and incubated at 37 °C. The insert-supported biofilms were transferred to a new 24-well plate with fresh TSB every 24 hours for a total of 72 hours of incubation. Afterwards, the insert-supported biofilms (referred to as Day 3 biofilms) were placed in wells containing 300 μL of PBS for 1 hour to remove excess TSB and then used in experiments as described or used to collect BCM. BCM was obtained by placing Day 3 biofilms in 24-well plates containing 300 μL/well HKGS supplemented-EpiLife® cell culture medium (no P/S) and incubated. Every 24 hours the medium was collected and replaced with fresh medium. A total of three 24-hour collections were pooled, stored at −20 °C, and used for experiments as BCM.

The number of viable bacterial cells in the initial *S. aureus* inoculum and the biofilms were determined using the spread plate technique. Briefly, samples were dissociated by vortexing and sonication, serially diluted in PBS, plated on tryptic soy agar (TSA), and incubated at 37 °C overnight. Afterwards, the plates were counted and the number colony forming units (CFU) per inoculum or insert was calculated. The collected BCM was also plated to ensure its sterility. If any bacterial growth was detected the medium was not used for experiments.

Planktonic-conditioned medium (PCM)

PCM was prepared to give a similar proportion of bacteria per unit fluid volume to that of the BCM. An overnight culture of *S. aureus* was grown in TSB at 37 °C with agitation. The cell suspension was then centrifuged at 3,000×*g*, and the cells were washed in PBS to remove excess TSB. The suspension was centrifuged again at 3,000×*g*, and the cells were resuspended in HKGS-supplemented EpiLife® medium (no P/S) at a cell density equivalent to a biofilm (5×10^9 CFU/mL). The final suspension was incubated at 37 °C with agitation for 24 hours. Afterwards, the suspension was filtered through a 0.22 μm filter, stored at −20 °C, and used for experiments as PCM.

Scratch assay

HK cultures were grown in 24-well plates (60,000 cells/well) for 2 days, after which 80–90% confluence was reached. The cultures were then scratched with a 200 μL plastic pipette tip, washed twice with HEPES-buffered saline (HBS), and resupplied with 300 μL of cell culture medium. The scratched cultures were imaged, obtaining the initial scratch area. Afterwards, the Day 3 biofilms were added to the HK cultures, and the *S. aureus*/HK cocultures were incubated in humidified 5% CO_2 incubator at 37 °C. This technique allowed diffusible factors produced by the biofilm to pass into the cell culture medium while excluding the bacteria themselves (Figure 1). Every 24 hours, the inserts were removed, the cells were rinsed with HBS, and the medium was replenished. The cultures were imaged again, the inserts were replaced, and the cocultures were returned to the incubator. The assay was terminated after 72 hours. Control HK cultures contained no biofilms, and all experiments were performed with triplicate samples. BCM and PCM were also used in the scratch assay using the techniques described.

Scratch assays were also conducted using antibiotics in the cell culture medium to determine if treating the biofilm would aid in scratch closure. The methods were similar to those previously described except P/S was used in the HKGS-supplemented EpiLife® medium.

All scratch images were taken using a ×2.5 objective on a Zeiss Axiovert S100TV microscope (Thornwood, NY). Images were analyzed and percent scratch area closed was calculated for each time-point using the Metamorph® image analysis program.

Staining and microscopy

HK were grown to 80–90% confluence on 18 mm^2 cover slips placed in six-well plates. Afterwards, the HK were exposed to 3 mL of BCM or PCM. After 24 hours of exposure, the HK were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 minutes at 37 °C followed by three 5 minute washes in PBS. Cells were then permeablized with 0.2% Triton-X 100 for 5 minutes at

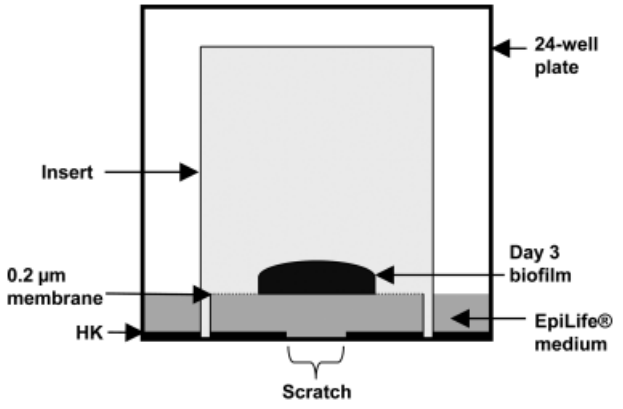

Figure 1. Schematic illustrating the *Staphylococcus aureus* biofilm and human keratinocyte (HK) coculture method. Note that the biofilm is resting on a 0.2 μm membrane and is not in direct contact with the HK.

room temperature, followed by three 5 minute washes in PBS. The HK were then stained for F-actin (BODIPY phalloidin, Invitrogen, Carlsbad, CA) and DNA (TO-PRO-3 Iodide, Invitrogen) using the manufacturer's instructions. The slides were then mounted using Vectashield® H-1000 (Vector Laboratories, Burlingame, CA) and imaged immediately. All images were imaged on a Leica TCS-SP2 AOBS confocal microscope with ×100 oil immersion objective unless otherwise noted. Control cultures were also stained and consisted of HK exposed to standard HKGS-supplemented EpiLife® medium (no P/S).

HK were also exposed to a series of solutions, including HKGS-supplemented EpiLife® medium, nonsupplemented EpiLife® medium, BCM, PCM, and Hank's buffered salt solution (HBSS, Hyclone) without calcium and magnesium and monitored continuously with light microscopy. Briefly, HK were grown to 80–90% confluence in 35 mm noncoated glass bottom Petri dishes (MatTek, Ashland, MA). Two hours before imaging, both the HK and the experimental solution were placed at room temperature to allow the temperature to equilibrate. Immediately before imaging, the cell culture medium was exchanged to the experimental solution and the cells were imaged using a ×16 water immersion objective. Bright-field images were captured every 45 seconds for 4 hours with an 18-ms exposure rate.

Keratinocyte viability

HK viability was assessed using 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide (XTT; Sigma, St. Louis, MO). To begin, 60,000 cells/well were seeded in 24-well plates and cultured for 2 days. Once 80–90% confluence was reached, HK cultures were exposed to BCM or PCM. At 3, 24, 48, and 72 hours, the conditioned medium was removed and replaced with 300 μL of fresh EpiLife® medium +60 μL of XTT. After 4 hours, the medium solution was sampled and the absorbance at 490 nm was measured. Afterwards, BCM or PCM was added to the wells, and the plate was returned to incubator until the next time point. Control HK cultures were exposed to standard HKGS-supplemented EpiLife® medium (no P/S). Triplicate cultures were used for all conditions.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining

TUNEL staining was used to investigate the induction of apoptosis. HK were grown to 80–90% confluence on 18 mm^2 cover slips placed in six-well plates. Afterwards, the HK were exposed to 3 mL BCM or PCM. At various time points, the HK were fixed in 4% paraformaldehyde in PBS for 15 minutes at 37 °C followed by three 5-minute washes in PBS. The APO-BrdU TUNEL Assay Kit (Invitrogen) was used following the manufacturer's staining protocol with one modification. The HK were not brought into suspension; rather, all enzyme solutions were made in the same proportions suggested by the manufacturer, and were added directly to the coverslip. The coverslips were then imaged using a Nikon Eclipse E800 epifluorescent microscope using a ×40 oil immersion objective. For analysis, three images of each condition were taken and the percentage of adherent cells staining positive for TUNEL was calculated. Control cultures were also stained and consisted of HK exposed to standard HKGS-supplemented EpiLife® medium (no P/S).

Statistical analysis

Data are presented as the mean ± standard deviation (SD). For two group comparisons, statistical analysis for significance was determined using as a two-tailed *t*-test assuming unequal variances with $\alpha=0.5$ and $p\leq0.05$ considered to be significant. For comparisons involving three or more groups statistical analysis for significance was determined using an ANOVA with a Tukey's HSD post hoc test with $\alpha=0.5$ and $p\leq0.05$ considered to be significant.

RESULTS

Biofilm and BCM

S. aureus biofilms were grown on tissue culture inserts. The initial inoculation of the insert was 7.25×10^5 CFU/insert, and after 3 days growth in TSB, the biofilm had grown to $1.52\pm0.45\times10^9$ CFU/insert (Figure 2). The biofilms were used in experiments or for the collection of BCM. One biofilm was used in one well containing 300 μL of supplemented-EpiLife® medium, which equated to cell density of $5.07\pm1.5\times10^9$ CFU/mL. The pH of the biofilm conditioned medium was 7.13 ± 0.02. The pH of the unconditioned medium was 7.20 ± 0.06.

PCM

Planktonic *S. aureus* was resuspended in supplemented EpiLife® at a cell density of 5×10^9 CFU/mL and incubated

Figure 2. Photograph of *Staphylococcus aureus* biofilm grown on a tissue culture insert. The insert (10 mm in diameter) is placed in a well of a 24-well tissue culture plate.

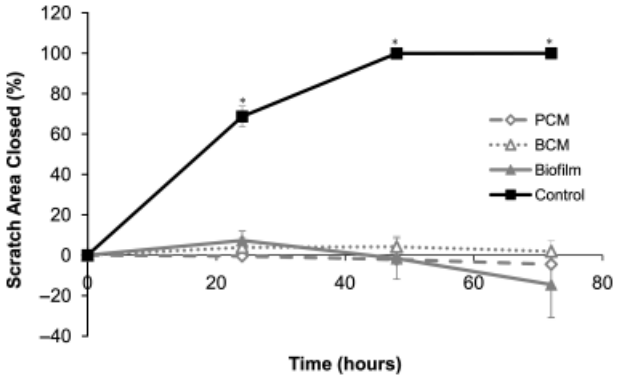

Figure 3. Percent scratch area closed for the in vitro scratch assay. Results are shown for three experimental groups where the human keratinocytes were exposed to either a *Staphylococcus aureus* biofilm, *S. aureus* biofilm-conditioned medium (BCM), or *S. aureus* planktonic-conditioned medium (PCM). The results for one control group are also shown. Results represented as mean ± standard deviation, $n=3$. *Significantly different from experimental groups at $p < 0.0001$.

for 24 hours at 37 °C with agitation. Before sterile filtration, the suspension was sampled, serial diluted, and plated to reveal a final bacterial cell density of 5.1×10^9 CFU/mL. The resulting PCM had a cell density equivalent to that of both the biofilm and the BCM. Thus, HK cultures exposed to the biofilm, BMC, or PCM were all in contact with medium conditioned by approximately 5×10^9 CFU/mL. After filtration, the pH of the PCM was adjusted from 6.81 ± 0.02 to 7.23 ± 0.01. The PCM was sterile filtered once again and stored at −20 °C until used.

Scratch assay

When *S. aureus* biofilms were cocultured with scratched HK cultures, the effect of biofilm exposure was evident after 24 hours. Scratches in control cultures were $68.7 \pm 5.1\%$ closed while biofilm-exposed cultures were $7.3 \pm 4.7\%$ closed ($p < 0.0001$, Figure 3). At 48 hours, the control cultures were $99.9 \pm 0.1\%$ closed, whereas the biofilm-exposed scratches had grown larger ($-1.6 \pm 10.2\%$ closed, $p < 0.0001$, Figures 3 and 4). By 72 hours, the scratches in the control cultures had closed, while the scratches in the biofilm-exposed cultures had expanded even further (100% vs. $-14.6 \pm 16.3\%$ closed, $p < 0.0001$, Figure 3). Similar results were obtained with the BCM. At all time points the scratched cultures receiving BCM had significantly less closure than the control group ($p < 0.0001$, Figures 3 and 4). The BCM were statistically equivalent to the biofilm-exposed group at all time points. Because the biofilm and BCM groups were equivalent, subsequent experiments were conducted using BCM.

Scratch closure in the PCM cultures was also similar to the biofilm and BCM culture. At all time points the cultures receiving PCM had significantly less closure than the control group ($p < 0.0001$, Figures 3 and 4). The PCM were statistically equivalent to the BCM and biofilm groups at all time points.

The assay was also used to assess the effect of antibiotics in combination with the biofilm on keratinocyte scratch closure. Using antibiotics in the cell culture medium decreased the size of the biofilm and allowed for partial scratch closure (Figure 5). The initial inoculum was 1.81×10^5 CFU/insert and after coculture the biofilm had reached $8.87 \pm 3.06\times 10^8$ CFU/insert. The use of P/S significantly decreased the biofilm to $2.31 \pm 1.89\times 10^4$ CFU/insert ($p=0.04$), and by 72 hours scratch closure had reached $24.5 \pm 4.5\%$ (Figure 5). Closure at the 48- and 72-hour time points was significantly greater than scratch closure without P/S ($p < 0.01$), but it was significantly less than controls ($p < 0.001$, Figure 5). There were no significant differences between control cultures using P/S and those without.

Staining and microscopy

Control, biofilm, BCM, and PCM-exposed HK were examined using both brightfield and confocal microscopy. After 24 hours, the biofilm-exposed cells looked markedly different than the control cells. The control HK maintained their typical cobblestone keratinocyte morphology (Figure 6A), whereas the biofilm-exposed cells were slightly rounded with pronounced borders between cells and pyknotic nuclei (Figure 6B). The PCM-exposed cells were beginning to lose their cobblestone morphology; cell bodies were round with increased angularity of the cell borders. The cells also had pyknotic nuclei and vaculated cytoplasm (Figure 6C).

Staining for F-actin and DNA revealed that the control HK remained in close contact with neighboring cells and exhibited organized actin cytoskeleton with a feathery appearance and were spread out on the glass surface (Figure 6D). Staining of the nucleus revealed densely staining nuclei with no nuclear fragmentation. However, HK in contact with BCM for 24 hours showed the formation of multiple dendrite-like extensions[13,14] and abnormal nuclear morphology (Figure 6E). Disorganization of actin structure was observed with the cells expressing dendrite-like extensions that were present predominantly at the adhesion surface between the coverslip and cell. The majority of the BCM-exposed cells also displayed fragmented nuclei. In contrast, the HK in contact with PCM for 24 hour displayed an organized actin-cytoskeleton with a slight feathery appearance and no dentrite-like extensions. There was also no evidence of nuclear fragmentation (Figure 6F).

The striking morphology displayed by the BCM-exposed cells in the scratch assay, prompted a more comprehensive investigation using time-lapse microscopy. After 3 hours of culture, the control HK retained their identifying keratinocyte shape and exhibited no extensions or nuclear condensation (Figure 7A). However, morphological changes were observed in the BCM-exposed HK; there was drastic cytoskeleton rearrangement with the formation of dendrite-like extensions (Figure 7B). The PCM cultured cells did not exhibit any morphological changes in the same 3-hour period (Figure 7C). The HK retained their characteristic keratinocyte morphology and they maintained contact with neighboring cells. There was no formation evidence of dendrite-like extensions.

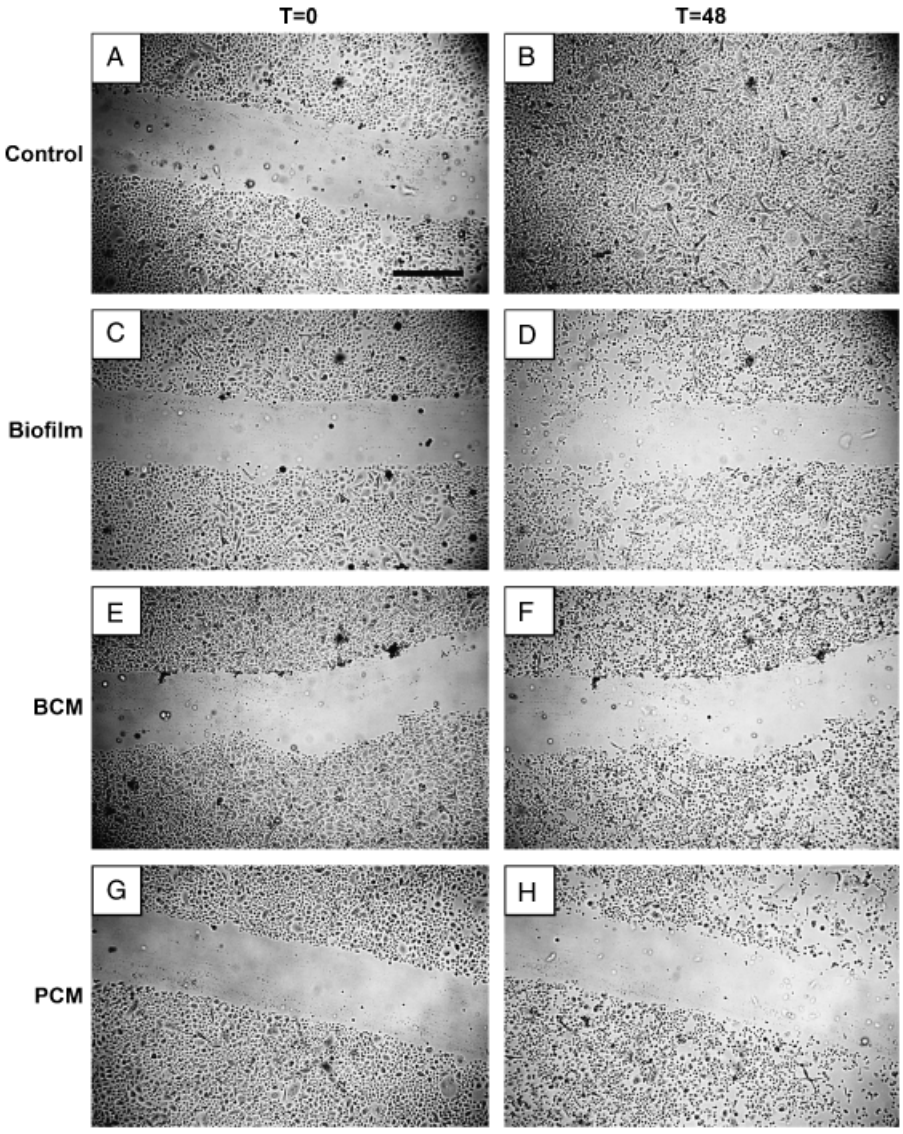

Figure 4. Bright-field images of in vitro scratch assay. (A) Control human keratinocytes (HK) culture immediately after scratching. (B) Scratched control HK culture after 48 hours. (C) HK culture that was exposed to *Staphylococcus aureus* biofilm. Image was taken immediately after scratching. (D) Scratched HK culture that was exposed to *S. aureus* biofilm for 48 hours. (E) HK culture that was exposed to *S. aureus* biofilm-conditioned medium (BCM). Image was taken immediately after scratching. (F) Scratched HK culture that was exposed to *S. aureus* BCM for 48 hours. (G) HK culture that was exposed to *S. aureus* planktonic-conditioned medium (PCM). Image was taken immediately after scratching. (H) Scratched HK culture that was exposed to *S. aureus* PCM for 48 hours. Scale bar represents 1 mm.

To ensure that the rapid effects of the BCM were due to biofilm-conditioning and not nutrient depletion of the medium by the biofilm, HK response to a series of control solutions was also investigated. Keratinocytes were exposed to EpiLife® medium without HKGS and HBSS, without calcium and magnesium. At the 3-hour time point, neither of the solutions elicited a response similar to the BCM (Figure 7D and E). The HK retained their characteristic keratinocyte morphology, maintained contact with neighboring cells, and did not produce dendrite-like extensions.

Keratinocyte viability

An XTT assay was employed to determine if there was a loss of cell viability post-BCM and PCM exposure. After only 3 hours of exposure to BCM, HK cultures exhibited reduced viability compared with the controls ($p=0.03$, Figure 8). The viability of the PCM cultures, however, were statistically equivalent to that of the control group ($p=0.86$, Figure 8). By 24 hours, the difference in viability between the groups had grown. Both the BCM and PCM groups displayed reduced viability compared with controls ($p\leq0.002$), but the BCM group showed a reduced viability compared with the PCM group as well ($p=0.05$). This trend continued through the 48- and 72-hour time points.

TUNEL staining

Biofilm exposure prevented scratch closure and reduced cell viability. Furthermore, post-biofilm exposure, the HK appeared rounded with condensed nuclei, suggesting that the cells were undergoing apoptosis. To explore this possibility, HK were exposed to BCM, and at selected time

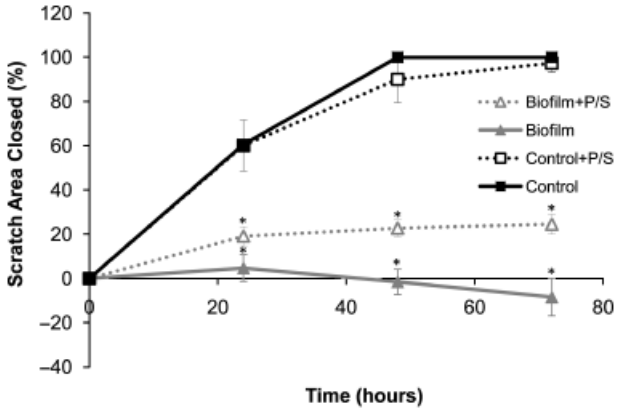

Figure 5. Percent scratch area closed for the in vitro scratch assay using antibiotics. Results are shown for two experimental groups where the human keratinocytes were exposed a *Staphylococcus aureus* biofilm or *S. aureus* biofilm and penicillin and streptomycin (P/S). The results for the two control group (medium with and without P/S) are also shown. Results represented as mean ± standard deviation, $n=3$. *Significantly different from controls at $p < 0.001$.

points were stained for TUNEL. HK were also exposed to PCM and standard HKGS-supplemented EpiLife® as a control. HK in contact with the BCM showed a steady increase in the number of adherent cells undergoing apoptosis over time with the vast majority of cells ($90.4 \pm 1.2\%$) succumbing to apoptosis after 48 hours of contact (Figure 9). Conversely, the HK in contact with PCM displayed a low, constant level TUNEL staining that was significantly lower than the BCM group ($p \leq 0.04$) from the 9-hour time point onwards. No TUNEL staining was observed in adherent cells in the control cultures. There were, however, detaching cells that stained positive for apoptosis in all conditions examined. The difference in staining between the BCM and controls at the 9-, 12-, 24-, and 48-hour time points, were significant (Figure 9, $p \leq 0.004$), whereas there was no significant difference between controls and the PCM group.

DISCUSSION

Few studies have investigated the effect of bacteria on epidermal cells. Of these, most involve the direct inoculation of mammalian cell cultures with planktonic bacteria. For example, researchers found that *S. aureus*[15,16] and *Salmonella typhi*[16] both invade and subsequently induce the necrotic and apoptotic death of keratinocytes. Group A *Streptococcus* cultured with keratinocytes also induced apoptosis.[17] Coculturing *Fusobacterium nuceatum* and keratinocytes increased keratinocyte migration.[18] Finally, after exposure to *A. actinomycetemcomitans*, HeLa and gingival keratinocytes displayed significant differences in gene regulation.[19] However, bacteria colonizing chronic wounds generally exist as biofilm, rather than planktonic, communities.[5,6,10] Before this investigation, there were no studies describing the effect of bacterial biofilms on mammalian cell cultures.

S. aureus has one of the highest incidence rates in traumatic, surgical, burn, and other wound infections.[20] Molecular analysis of chronic wounds also revealed *S. aureus* to be a principal component of the microbial infection.[2,21] It is, therefore, clinically relevant to investigate the effects of *S. aureus* biofilms on the cells involved in wound healing, specifically reepithelialization. In this investigation, *S. aureus* biofilms were grown from a clinical chronic wound isolate and grew to approximately 1×10^9 CFU. Clinically, wounds presenting with a level greater than or equal to 10^6 CFU of any organism per gram of tissue is associated with impaired healing.[8]

The scratch assay is a simple wound model system, lacking extracellular matrix and inflammatory components. However, it is used extensively because it allows for the direct investigation of external agents on the cells involved in wound healing.[22–26] In this investigation, *S. aureus* biofilms were found to significantly reduce scratch closure (Figure 3). The presence of a biofilm in culture with HK actually increased the initial scratch size. However, minimal scratch closure was recovered when the biofilm was treated with antibiotics (Figure 5).

An increase in scratch size was also obtained with BCM and PCM. HK in BCM for 24 hours had an appearance similar to those cultures in the presence of a biofilm with the formation of dendrite-like extensions and fragmented nuclei in the HK (Figure 6). Time-lapse microscopy revealed that the distinctive morphology displayed by the biofilm/BCM-exposed cells was apparent after only 3 hours of exposure. The PCM-exposed cells, however, did not form dentrite-like extensions at neither the 3- nor 24-hour time points, suggesting the morphological changes observed in the biofilm/BCM groups were unique. Furthermore, the BCM group also displayed significant reduction in cell viability and a significant increase in TUNEL staining compared with both the PCM and control groups. Several studies have shown that coculturing planktonic *S. aureus* with keratinocytes induces apoptosis; the keratinocytes first internalize the bacteria[27] and then undergo apoptosis.[15,16] These consequences, however, require the *S. aureus* to be direct contact with the keratinocytes. The results presented herein suggest that *S. aureus* biofilms can induce HK apoptosis without prior internalization.

The biofilm, BMC, and PCM groups were all normalized to bacterial cell number. HK exposed to a biofilm, BMC, or PCM, were all subjected to medium conditioned by approximately $5.07 \pm 1.5 \times 10^9$ CFU/mL. Thus, the viability and apoptosis differences observed by the biofilm and BCM-exposed HK cultures suggested that the biofilm either supplemented or depleted the culture medium of a causative agent. To investigate nutrient depletion, HK were exposed to a series of solutions and examined continuously via time-lapse microscopy. The solutions selected were chosen to represent environments depleted of selected nutrients. The exact formulation of EpiLife® medium is considered proprietary by the manufacturer. Therefore, solutions mimicking the medium but lacking certain ingredients were not made. Rather, available solutions were used to simulate nutrient depleted environments. EpiLife® medium without the HKGS supplement was depleted of growth supplements (bovine pituitary extract, bovine

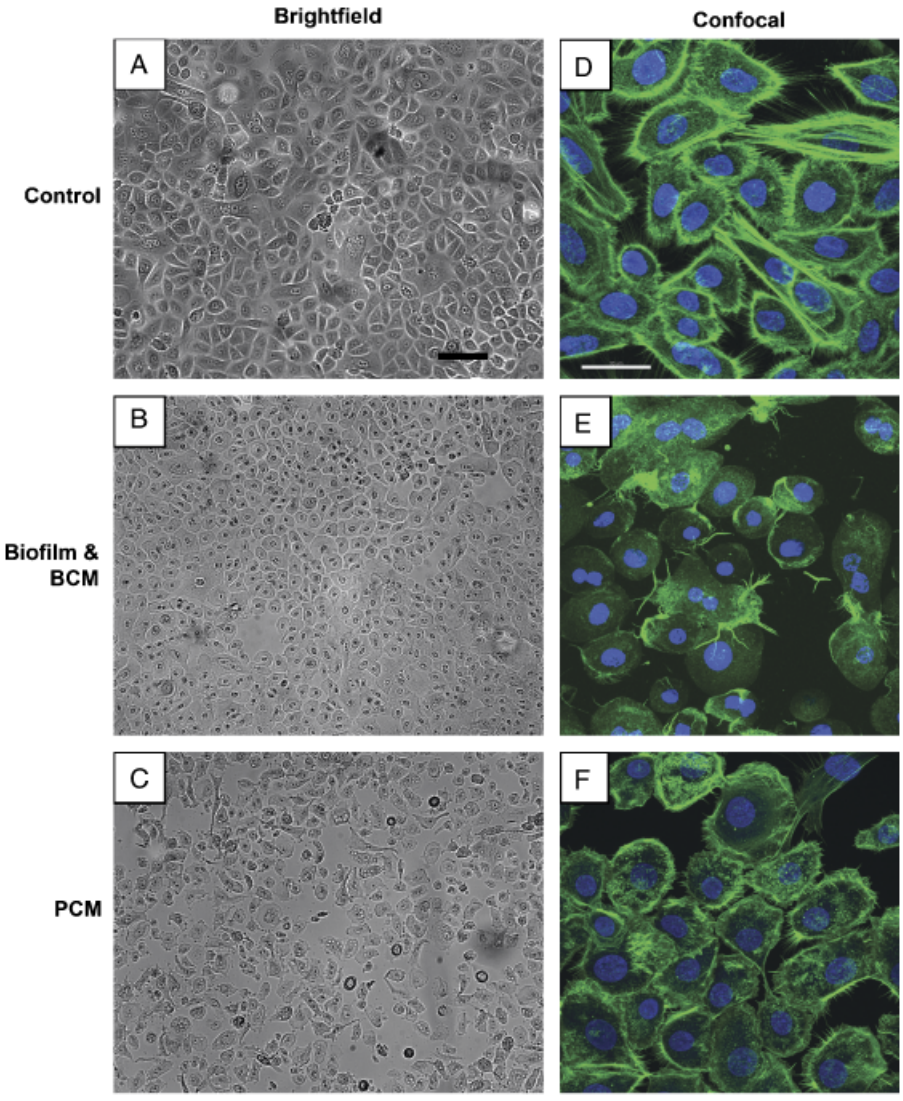

Figure 6. Images of human keratinocytes (HK). (A) Bright-field image of control HK at 24 hours. (B) Bright-field image of biofilm-exposed HK at 24 hours. (C) Bright-field image of PCM-exposed HK at 24 hours. Scale bars for the brightfield images represent 100 μm. (D) Confocal image of control HK stained for F-actin (green) and the nucleus (blue). (E) Confocal image of HK exposed to biofilm-conditioned medium (BCM) for 24 hours. The cells are stained for F-actin (green) and the nucleus (blue). (F) Confocal image of HK exposed to planktonic-conditioned medium (PCM) for 24 hours. The cells are stained for F-actin (green) and the nucleus (blue). Scale bars for the confocal images represent 30 μm.

insulin, hydrocortisone, bovine transferrin, and human epidermal growth factor). HBSS was used to represent an environment depleted of growth supplements, amino acids, and vitamins. After 3 hours of exposure, the cells in contact with the solutions retained their characteristic HK morphology (Figure 7), and there was no reduction in cell viability (data not shown).

Keratinocytes are particularly sensitive to extracellular calcium levels.[28] An increase in extracellular calcium can lead to the rapid redistribution of desmoplakin and rearrangement of actin filaments.[29] Elemental analysis using inductively coupled plasma mass spectrometry of BCM and unconditioned medium did not show a difference in calcium levels (data not shown). Futhermore, there was no difference in pH. Thus, the investigations described herein suggest that the results obtained the biofilm-exposed HK could not be attributed to changes in calcium levels, pH, or nutrient depletion, suggesting that the *S. aureus* biofilms produced a causative agent.

S. aureus produces a number of toxins including toxic shock syndrome toxin-1 (TSST-1) and staphylococcal enterotoxins A-E, the exfoliative toxins, and leukocidin.[30] These toxins do not possess direct cell damaging action, but have a potent effect on cells of the immune system by inducing the overproduction of cytokines.[31] Hemolysins α–δ, nucleases, proteases, lipases, hyaluronidases, and collagenase are also produced by *S. aureus*.[30] The lysis-inducing toxins directly damage host cells by generating pores in the plasma membrane, while the enzymes help degrade host tissues.[30] Staphylococcal α-toxin has been found to induce profound keratinocyte cytotoxicity,[31] and it has been suggested that α-toxin is required for *S. aureus* biofilm formation.[32] Researchers have also found the cell surface-expressed fibronectin-binding proteins (FnBPs) on *S. aureus* inhibit keratinocyte migration.[22] While there are no reports of *S. aureus* secreting FnBPs, it is possible that the proteins were in both the BCM and PCM and contributed to the failure of the HK to migrate. Current studies are

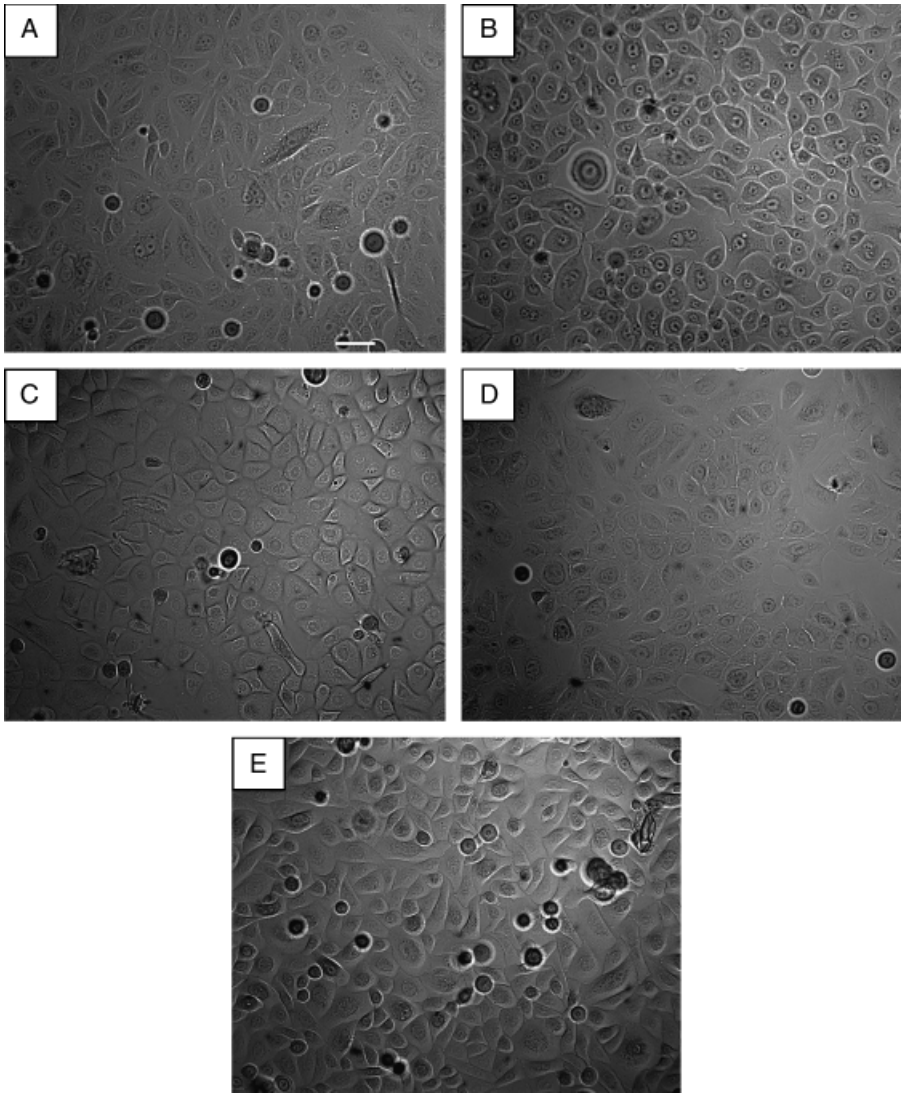

Figure 7. Bright field images of human keratinocytes (HK) exposed to different solutions for 3 hours. (A) Control cells. HK were exposed to EpiLife® medium supplemented with human keratinocyte growth supplement (HKGS). (B) HK exposed to biofilm-conditioned medium . (C) HK exposed to planktonic-conditioned medium. (D) HK exposed to EpiLife® medium without HKGS supplementation. (E) HK exposed to Hank's buffered salt solution. Scale bar represents 50 μm.

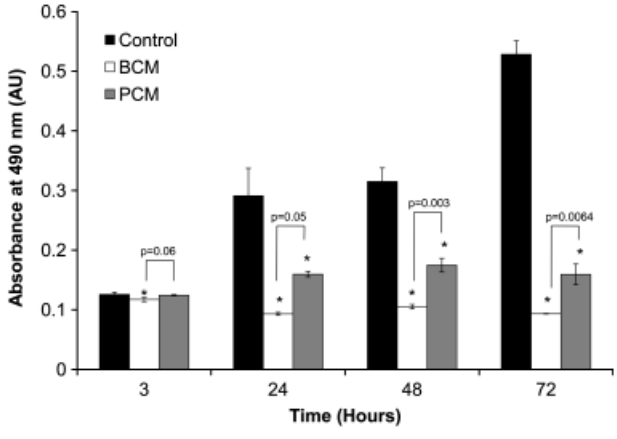

Figure 8. XTT cell viability assay. Absorbance at 490 nm of human keratinocytes exposed to biofilm-conditioned medium (BCM) or control medium. Results represented as mean ± standard deviation, n=3. *Significantly different from controls at $p < 0.05$.

focusing on identifying causative agent(s) for influencing both keratinocyte migration and viability in this biofilm model system.

The microbiology of chronic wounds has been actively studied in recent years.[20] While the presence of microbial biofilms has been established[10] few studies illustrate the role of biofilms in chronic wound pathogenesis. The coculture model described herein allows the study of host-biofilm interaction. Furthermore, the coculture model can be used as a tool in the development and evaluation of antibiofilm therapeutics. Treatment effect on the biofilm, the cell culture, and on *in vitro* scratch closure can be evaluated. For example, the addition of antibiotics to the cell culture media significantly reduced the size of the *S. aureus* biofilm and significantly increased wound closure compared with the coculture without antibiotics.

The care of chronic wounds poses enormous material and patient costs. If a fraction of these cases stem from biofilm infection, the development of effective antibiofilm treatments that that accelerate wound healing would be significant.

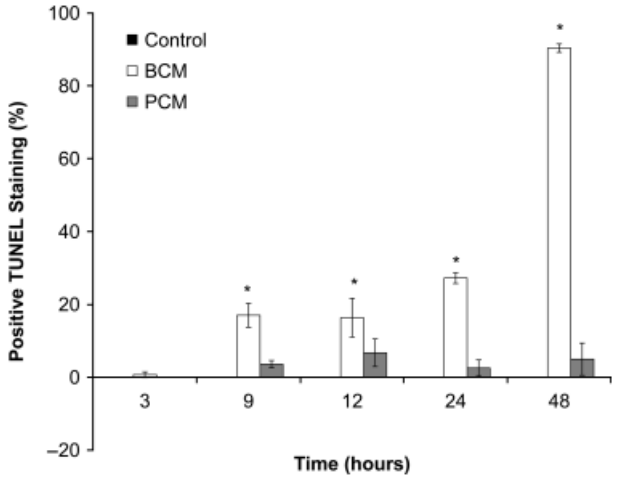

Figure 9. Percentage of human keratinocytes cultures staining positive for terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). Note: The control cultures had zero positive TUNEL staining in adherent cells at all time points. Results represented as mean ± standard deviation, $n=3$. *Significantly different from controls at $p < 0.005$.

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