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Chronic Wound Biofilm Model

Kasturi Ganesh, MD

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*Correspondence: Department of Surgery, Comprehensive Wound Center, Davis Heart and Lung Research Institute, The Ohio State University Medical Center, Columbus, OH 43210 (e-mail: kasturi.ganesh@osumc.edu).

Kasturi Ganesh, Mithun Sinha, Shomita S. Mathew-Steiner, Amitava Das, Sashwati Roy, and Chandan K. Sen*

Department of Surgery, Comprehensive Wound Center, Davis Heart and Lung Research Institute, The Ohio State University, Columbus, Ohio.

Significance: Multispecies microbial biofilms may contribute to wound chronicity by derailing the inherent reparative process of the host tissue. In the biofilm form, bacteria are encased within an extracellular polymeric substance and become recalcitrant to antimicrobials and host defenses. For biofilms of relevance to human health, there are two primary contributing factors: the microbial species involved and host response which, in turn, shapes microbial processes over time. This progressive interaction between microbial species and the host is an iterative process that helps evolve an acute-phase infection to a pathogenic chronic biofilm. Thus, long-term wound infection studies are needed to understand the longitudinal cascade of events that culminate into a pathogenic wound biofilm.

Recent Advances: Our laboratory has recently published the first long-term (2 month) study of polymicrobial wound biofilm infection in a translationally valuable porcine wound model.

Critical Issues: It is widely recognized that the porcine system represents the most translationally valuable approach to experimentally model human skin wounds. A meaningful experimental biofilm model must be *in vivo*, include mixed species of clinically relevant microbes, and be studied longitudinally long term. Cross-validation of such experimental findings with findings from biofilm-infected patient wounds is critically important.

Future Directions: Additional value may be added to the experimental system described above by studying pigs with underlying health complications (*e.g.,* metabolic syndrome), as is typically seen in patient populations.

INTRODUCTION

Wound infection is a major contributor to wound chronicity.[1] Wounds are considered chronic if they take more than 4 weeks to heal.[2] Persistent infection may not only arrest growth of the repairing tissue but it is also known to substantially modify the inflammatory response compromising timely resolution.[3–5] The influence of wound infection on the healing process may depend on the following factors: (1) wound etiology, dimension, tissues involved, and anatomical location,[6,7] (2) host factors and response,[8,9] (3) composition of polymicrobial species,[10,11] and (4) state of infection, that is, planktonic and/or biofilm. Biofilm-infected wounds suffer from compromised closure.[12]

What are clinically presented biofilms?

Biofilms refer to a structurally distinct state of microbial infection, where microbes are encased in an extracellular polymeric substance produced by microbes. In the clinical presented form, biofilms are host interactive and polymicrobial, often including fungi, viruses, and/or

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protozoa in addition to multispecies bacterial communities. Biofilms are self-assembling, self-sustaining, and function as cohesive sessile entities tolerant to antimicrobial therapies.

Models of biofilm infections

Biofilms have been implicated in numerous acute and chronic infections.[13–15] Several reports have linked biofilms to the induction and persistence of inflammation and delayed healing in wound infections.[1,9,15–21] In addition, studies (using a combination of traditional culture methods, microscopic analyses, and molecular techniques) involving wound samples from human patients support the presence of mixed populations of microorganisms in different types of chronic wounds.[9,11,13,18,19,22,23] However, despite several studies linking biofilm infection to delayed wound healing, the mechanisms and significance of biofilms in wound infection remain poorly understood. Dynamic interactions between multiple species identified within wound biofilms and their exact role in delaying wound healing represent yet another void in our understanding. In addition, the intricate details of the interactions between the host immune system and the biofilm invader remain to be explored *in vivo*. A significant limiting factor in investigating wound biofilms is the availability of appropriate chronic models of skin wound biofilm infection, where longitudinal assessments of cascading mechanisms may be studied over time.

The study of biofilms has been *in vitro* based for decades.[24] Studies on animals, including rats, mice, rabbits, and pigs, have mostly addressed short-term acute-phase processes ranging between 2 and 26 days of infection (Table 1).[7,10,25–29] Such approaches are of limited value as they fail to capture the long-term interplay between the host and biofilm, which has a significant bearing of the wound microenvironment at the site of the infection.[17] This article aims to concisely and critically review the various *in vitro* and *in vivo* models used for the study of biofilm infections of wounds with specific emphasis on the preclinical porcine model of chronic infections (duration of 8 weeks) recently reported by our laboratory.[12]

IN VITRO BIOFILM MODELS

In an effort to study complex communities of clinically relevant bacteria under controlled conditions, numerous *in vitro* model systems have been developed.[30,31] Most of these utilize abiotic surfaces to study biofilm growth and are broadly classified as closed/batch or open/continuous systems based on the approach of nutrient supply. These include microtiter plate models such as the Calgary biofilm device,[30,31] flow displacement or bioreactor models such as the modified Robbins device,[30,31] and the CDC bioreactor and microfluidic devices such as the Bioflux systems.[30,31] In addition, *in vitro* cell culture-based models employing biotic surfaces such as reconstituted human epithelia[30] have been used to study the interactions of cells with biofilms.[30] The Lubbock model (the arguably presented chronic wound model) is useful to study interactions between multiple microbes isolated from clinical wounds, including anaerobic species in the biofilm.[32–34] However, it is important to recognize that this approach is *in vitro* and not applicable to the study of dynamic biofilm–host interactions *in vivo*. The Lubbock model may be useful to test the efficacy of antimicrobial agents against biofilm versus planktonic microbes. A variation of this model studied the growth of biofilms in the absence of a solid surface.[35] Given the microenvironmental complexities of a chronic wound matrix, recreating it experimentally is a major challenge. Some *in vitro* models of biofilm infection have utilized tissue-engineered skin equivalents, such as Graftskin™, which possess histological parallels compared to human skin.[36]

Most of these *in vitro* models have addressed two main suspects in chronic wound biofilms—*Staphylococcus aureus* and *Pseudomonas aeruginosa*. Undoubtedly, these *in vitro* models have improved our understanding of intercellular communication involving the quorum-sensing system, mechanisms of antimicrobial tolerance, and the efficacy (or lack thereof) of various therapeutic measures. They are good supplemental approaches to delineate underlying molecular and cellular mechanisms of biofilm formation and function. However, they do not address the iterative host response component and are therefore significantly limited in their ability to provide clinically relevant information. Although supplements may be added to the media with the intent to recapitulate the wound milieu,[36] it is important to recognize that such efforts will always fall much short of reconstituting the *in vivo* chronic wound microenvironment. Furthermore, the lack of a host interaction component in such approaches must be acknowledged while interpreting any finding in the context of wound infection. Of note, wound biofilm biology is not just about biofilm alone, dynamic exchange between the microbial biofilm and host responses defines the biofilm itself.

Table 1. In vivo *biofilm models*

No.	Authors	Host species	Bacterial species (mono- or multispecies)	Duration of study postbacterial inoculation	Comments
1	Roy *et al.*[12]	Pig	*Pseudomonas aeruginosa* and *Acinetobacter baumanii* (multispecies)	56 days	Full-thickness burn wounds in pigs were infected with multispecies (*P. aeruginosa* and *Acinetobacter baumanii).* This work establishes the first chronic preclinical model of wound biofilm infection aimed at addressing the long-term host response and demonstrated compromised skin barrier functions.
2	Zhao *et al.*[27]	Mouse	*P. aeruginosa* (monospecies)	26 days	First biofilm model in diabetic condition. Full-thickness circular punch wound biopsies were made on dorsum of the mice and challenged with *P. aeruginosa* (monospecies) This work determined the significant delay in wound healing compared with unchallenged control mice.
3	Watters *et al.*[7]	Mouse	*P. aeruginosa* (monospecies)	16 days	Diabetic condition was induced by administering streptozotocin. Excisional wounds were inoculated with *P. aeruginosa*. This work suggests that the diabetic wound environment may promote the formation of biofilms.
4	Dalton *et al.*[10]	Mouse	*Staphylococcus aureus, P. aeruginosa, Enterococcus faecalis,* and *Finegoldia magna* (multispecies)	12 days	*P. aeruginosa* became the dominate species over time demonstrating interspecies competition. The wound closure delayed in multispecies-infected group compared to the monospecies-infected group.
5	Gurjala *et al.*[40]	Rabbit	*S. aureus* (monospecies)	10 days	Full-thickness circular wounds were made in the ears of New Zealand white Rabbits and subsequently infected with *S. aureus*. Wound healing outcome studied. First model where biofilm was challenged with antimicrobials.
6	Simonetti *et al.*[52]	Mouse	*S. aureus* (monospecies)	7 days	First model to measure wound healing outcome in presence of biofilm.
7	Nakagami *et al.*[37]	Rat	*P. aeruginosa* (monospecies)	7 days	To address chronicity of wounds, the authors developed pressure-induced ischemic wound model.
8	Pastar *et al.*[47]	Pig	*MRSA* and *P. aeruginosa* (multispecies)	4 days	Partial-thickness wounds were infected with *methicillin-resistant S. aureus, P. aeruginosa,* and mixed infection to each animal group. This study underlines the importance of bacterial interactions in multispecies wound infections demonstrating that synergy can alter the virulence resulting in impaired healing of wound.
9	Apidianakis and Rahme[53]	Drosophila	*P. aeruginosa* and other bacterial species studied (monospecies)	4 days	Pin-pricked wounds were made on the back of *Drosophila melanogaster* and subsequently infected with bacteria.
10	Davis *et al.*[25]	Pig	*S. aureus* (monospecies)	2 days	Partial-thickness wounds in pigs were infected with *S. aureus*. The *in vivo* antimicrobial treatment demonstrated increased antimicrobial resistance when compared with their planktonic phenotype.
11	Akiyama *et al.*[54]	Mouse	*S. aureus* (monospecies)	60 h	Incisional dorsal wounds on mice were infected with *S. aureus*. Identification of biofilm glycocalyx through electron microscopy.
12	Rashid *et al.*[55]	Mouse	*P. aeruginosa* (monospecies)	24 h	Burned wound model on mice to study the role of polyphosphate kinase gene (PPK) in the virulence and quorum-sensing mechanism of bacteria.

IN VIVO BIOFILM MODELS

The notion that bacterial biofilms may underlie wound chronicity and persistence is gradually gaining wider acceptance.[1,3–5] Therefore, there is heightened interest to understand the progressive iterative interaction between the biofilm and the host response in the healing wound. Clinically presented relevant wounds are, for the most part, chronically infected denying the opportunity to address iterative mechanisms that come into play as the wound is infected and progresses to chronicity. Voluntary infection of human acute wounds by pathogenic bacteria is beyond the scope of ethical limits. Thus, evolving host-microbial processes may be only studied in an appropriate preclinical model. Such studies would help understand how biofilm infection may potentially derail the otherwise helpful inflammatory process resulting in chronic inflammation and pathological wound closure. Although microbial biology may be more easily studied *in vitro* or *ex vivo*, it is questionable whether such studies capture microbial mechanisms that are only unleashed in response to host interaction. *In vivo* biofilm models have included

the study of invertebrates such as *Drosophila melanogaster* and *Caenorhabditis elegans* (used to study *Pseudomonas*, *Staphylococcus*, or *Yersinia* monospecies biofilms) and numerous vertebrates such as rats, mice, rabbits, and pigs (Table 1).[30] Each model has its own advantages and disadvantages, some better than others as it relates to capturing the complexities of wound infection.

At present, much of our understanding of host responses to biofilm infection in wounds is derived from rodent models of wound healing using single-species biofilm infections (particularly involving *S. aureus* or *P. aeruginosa*). Among the so-called chronic models is a rat pressure-induced ischemic wound model (7 days) and genetically or chemically induced murine diabetic model (14–26 days). These models possess the inherent advantages of an *in vivo* setting, but suffer from some limitations related to the approach adopted.[27,37,38] First, it is well known that wound healing in rats and mice is limited in their ability to represent human skin wound healing, particularly because rodent cutaneous wound close primarily by contraction. This limitation may be addressed by the use of splinted wounds to recapitulate the granulation and re-epithelialization somewhat comparable to human wound healing. Second, very few studies using these models have attempted to recapitulate the polymicrobial nature of wound infections.[10,39] Third, majority of these studies have been short-term acute-phase studies that are insufficient by design to understand the long-term implications of biofilm–host interactions. Among small animals, the rabbit ear wound model seems promising. Outcomes such as impairment of epithelialization, overabundance of granulation tissue, and a hyperinflammatory state are interesting.[8,26,40–43] However, reported studies involve short-term infection disallowing prolonged interaction between polymicrobial pathogens and the host. In that respect, the rabbit ear model suffers from limitations comparable to those discussed for the rodent models.

PORCINE MODELS

It is widely accepted that porcine skin wound healing most closely resembles the human healing process. Anatomically, porcine skin shows high homology with the human skin. A review of 25 wound therapies revealed that porcine studies were in agreement with humans 78% of the time compared to 53% and 57% with rodents and *in vitro*, respectively.[44] With respect to translational value, the Wound Healing Society recommends the porcine model as the most relevant preclinical model of skin wound healing.[45] Additionally and importantly, the human immune system has a higher similarity to the porcine immune system compared to rats or mice, making it a better suited model for studies on the host interactions that are integral to the complexities of the pathological biofilm in wound infections.[46] Davis *et al.*[25] developed a porcine wound biofilm model, where partial-thickness wounds in pigs were infected with *S. aureus*. Using electron microscopy, the presence of biofilm matrix was established. This work also demonstrated that biofilms were nonresponsive to standard antimicrobial therapies. However, in this work, wound healing outcomes were not addressed. Furthermore, this was a short-term study where the infection lasted for only 2 days. Polymicrobial infection with *S. aureus* and *P. aeruginosa* has been tested on the porcine skin wound model. This was also a short-term study lasting for 4 days, which does not allow for the iterative microbe–host interplay toward a mature biofilm relevant to those present in chronic wounds.[47]

All currently reported porcine models addressing biofilm infection address short-term acute-phase responses and therefore limited in power to understand long-term clinically relevant host–biofilm interaction.[25,45,46] Our interest in understanding the host response to chronic infection necessitated the development of a wound infection model that recapitulates the persistent nature of these types of wounds. Given the widely acknowledged advantages provided by the pig as an experimental system to study wounds, we developed polymicrobial biofilm infection on full-thickness burn wounds.[12] In the model, host–microbe interactions were studied for 8 weeks, during which we noted the unfolding of a cascade of events resulting in deficits in the barrier function of the repaired skin. This burn wound biofilm satisfies the criteria of an established biofilm, as proposed by Parsek and Singh.[48] The biofilm was surface adherent, bacteria that existed in cell clusters or microcolonies encased in the extracellular matrix, persistent and localized over 4 weeks, and resistant to antimicrobial treatments despite the fact that the responsible organisms are susceptible to the same antimicrobials in the planktonic state.[12,48] Furthermore, biofilm infections are often present in the host tissue for extended periods, during which time they may compromise the host response to injury. In a sessile biofilm style of living, bacteria attain unexampled phenotypes by regulating gene expression that supports biofilm biology.[49] Whereas there is no known biofilm biomarker gene

identified for *P. aeruginosa*, we evaluated the expression patterns of some genes previously studied under biofilm growth conditions. These included *rpoS*, which is implicated in the morphology and antibiotic tolerance of biofilms,[49] and *rhlR*/*aprA*, previously linked to quorum sensing and biofilms.[50,51] The expression of *rhlR*, *rpoS*, and *arpR* was significantly upregulated in our biofilm system.[12] This recent work from our laboratory is the first to provide insight into the progressive development of host–microbe interactions resulting in loss of barrier function of the repaired skin.[12]

TAKE-HOME MESSAGES

- Bacterial biofilms impair wound healing.
- While biofilm infection may or may not influence wound closure as assessed visually, it compromises the barrier function of the repaired skin.
- Biofilms are defined by a progressive iterative interplay between hosts and microbes. Thus, the study of explant tissues lacking the immune response system is of limited value.
- Long-term (>4 weeks) polymicrobial infection of *in vivo* porcine wounds represent the most translationally valuable approach to study wound biofilm.
- Rodent studies involving genetically modified animals may be useful to extend observations from human and porcine studies such that mechanistic pathways are delineated

Our work provides first evidence demonstrating that biofilm infections induce microRNAs in the host tissue that silence the function of tight junction proteins critical for the proper maintenance of skin barrier function. Importantly, this has led to the novel observation that although visual inspection of the wound (current clinical standard) indicates wound closure, transepidermal water loss measurements for skin barrier integrity indicate that the biofilm-infected wounds undergo a pathological repair process where the skin closing the wound is faulty. A functionally compromised epidermal barrier could make the wound vulnerable to repeated infections, resulting in postclosure complications. This observation therefore drives home the importance of functional assessments of skin barrier functions in addition to the current clinical standard in monitoring wound healing progression. It also opens up new avenues for intervention strategies targeting microRNAs with the goal to restore normal barrier function.

SUMMARY

A variety of model systems have helped broaden our understanding of the role of bacterial biofilm infections in the regulation of wound healing. Studies in rodent models may be powerful in providing a mechanistic insight. However, their translational relevance remains limited. Long-term polymicrobial biofilm infection on porcine wounds may be considered as being powerful with respect to translational value. Observations from this model may be further studied in genetically modified rodents to elucidate underlying molecular mechanisms. Short-term infection studies are of limited value because they capture acute response and are not powered to study the progressive and iterative host–microbe interplay that is critically important in defining the clinically presented biofilm infection.

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ABOUT THE AUTHORS

Kasturi Ganesh, MD, is currently a postdoctoral fellow at the Ohio State University Medical Center (OSUMC). Her research is focused on the development of large animal pre-clinical model for mixed infection biofilms that complicate wound healing. **Mithun Sinha, PhD,** is a postdoctoral fellow at OSUMC where he is currently working on the role of small noncoding RNAs, especially microRNAs in wound healing outcomes. **Shomita S. Mathew-Steiner, PhD,** is a OSUMC postdoctoral fellow investigating aspects of mixed-species bacterial biofilms in wound healing in a large animal model of metabolic syndrome. **Amitava Das, MPharm,** is a graduate student at OSUMC and is studying the role of macrophages in the resolution of inflammation during diabetic wound healing. **Sashwati Roy, PhD,** is an Associate Professor of Surgery and Director of Laser Capture Molecular Analysis facility at OSU. She is an expert in the significance of macrophages and inflammation in chronic wounds. **Chandan K. Sen, PhD,** is Professor and Vice Chair of Research of Surgery, Executive Director of the OSU Comprehensive Wound Center and Director of the OSU Center for Regenerative Medicine and Cell Based Therapies. He serves as a program director (Innovation and Collaboratory) for the OSU Clinical and Translational Science.

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