How bacterial biofilms affect chronic wound healing: a narrative review

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Abstract

Bacterial biofilm is a formidable influencing factor affecting healing of chronic wound. The mechanisms are as diverse as the bacterial species contained in the biofilm. In an altered environment the biofilm accommodates polymicrobial colonies in which microorganisms undergo phenotypic and genetic changes to sustain adverse environmental influence. Biofilm bacteria inhibits cell proliferation, prevents cell migration and cause cell kill in a number of ways. It is believed that different bacterium has different mode of action through elaboration of various bioactive factors leading to persistence of low-grade inflammation in the wound bed which is predominantly bacteria-centric. Their ability to survive in low oxygen tension allows them to persist in relatively hypoxic conditions in the wound bed and in presence of increased bioburden. Although the biochemical mechanisms of biofilm influence on chronic wound healing are unfolding slowly, it is difficult to develop clinical studies due to ethical concerns. Therefore, most of the accumulated evidence are based on animal models and in vitro studies. This narrative review attempts to focus on the various mechanisms responsible for delayed healing in presence of bacterial biofilms.

Keywords: Biofilm, Chronic wound, Delayed healing, Wound healing, Bacteria, Biofilm bacteria

It is amazing how a complex event like wound healing is excellently orchestrated by cells and cytokines into a dynamic yet simple process. Four distinct phases such as hemostasis, inflammation, proliferation, and maturation are recognized normally with some temporal overlap and smooth transition. A number of extrinsic and intrinsic factors seem to influence the process of healing, which under normal circumstances remain in a perfect balance. Persistent insult may lead to delayed healing and a chronic wound formation, which by definition is > 6 weeks duration. Most of these chronic wounds are arrested in the inflammatory phase of healing. A prolonged inflammatory phase not only delays the onset of proliferation, but high levels of inflammatory mediators and proteases also disrupt the normal process of proliferation and epithelialization.

Bacteria are ubiquitous in nature and most acute cutaneous wounds would heal in the presence of normal skin flora. Infection starts when the balance between bacterial dose, virulence and immunity tilts in favor of micro-organisms. Initially it was believed that wound infection is the result of colonization and invasion of planktonic bacteria which reside freely in the environment, and the concept of contamination, colonization and infection as a sequential process was proposed. However, molecular sampling techniques have shown that chronic wounds harbor a more diverse population of bacteria than was thought of before and research in the last couple of decades has established the role of biofilm as a more comprehensive pattern of bacterial influence on chronic wound healing.

The nature of biofilm

Biofilms are complex colonies of bacterial populations enmeshed in a protective extracellular polymeric substance (EPS), which helps them to adhere to a suitable surface. Although it can be formed by many species, Pseudomonas aeruginosa and Staphylococcus aureus, the 2 very common microbes responsible for wound biofilm, have been studied the most. As it is unethical to experimentally generate biofilms in human, most of the present knowledge has been derived from in vitro and animal studies. A meta-analysis of published data indicates a 78.2% prevalence of biofilms in chronic wounds, making these a significant threat to healing. In the outer layers of a biofilm, bacteria are mostly aerobic, are actively metabolizing, multiply rapidly and grow easily in cultures. The deeply situated bacteria can survive in low oxygen tension or anaerobic environment, remain dormant for long time and poorly grow in conventional cultures. Definite genetic changes alter their phenotype and result in their poor recognition by the Toll-like receptors (TLR) of innate immune system. The EPS also provides a physical barrier to immune attack and drugs. The resident bacteria communicate with one another through quorum sensing (QS) molecules. Some of these also act as bacterial virulence factors and protect the colony against immune attack. Clinically, a chronic wound with red, friable granulation tissue covered by a slimy layer that comes back after debridement, with increased exudate formation and having evidence of a receding epithelial margin, is likely...
to be harboring a biofilm\textsuperscript{[16]} It may or may not be associated with slough, which is dead necrotic tissue at the wound bed\textsuperscript{[17]}. Currently biofilm is considered as an independent factor causing delayed wound healing\textsuperscript{[18]}. Biofilm delays wound healing

In a diabetic mouse model with \textit{P. aeruginosa} biofilm Zhao et al\textsuperscript{[19]} demonstrated delay in wound healing when compared with the control mice group without biofilm. The wounds with biofilm had thick epidermis and dermis, poorly vascularised matrix and showed evidence of delayed epithelialization. These features closely mimicked those of human chronic wounds, in which matrix becomes unhealthy and epithelial margins get thickened, hyperkeratotic and form epibole\textsuperscript{[20]}. Subsequently in their later research, Zhao et al\textsuperscript{[3]} compared chronic wounds with \textit{P. aeruginosa} biofilms in diabetic mice with a control group without biofilms in terms of wound closure, histology, gene expression, and blood glucose levels. There was a significant delay in healing (97\% vs. 56\%) at 4 weeks with an overall average delay of 2 weeks in the biofilm group. A 10-fold increase in interleukin 1\(\beta\) and interleukin 6 (IL-1\(\beta\), IL-6) and matrix metalloprotease-10 (MMP-10) expressions was observed at fourth week, which indicated persistent inflammation\textsuperscript{[21]}. High levels of MMP-10 was suggestive of ongoing collateral matrix damage resulting in delayed healing\textsuperscript{[22]}. Interestingly, expression of tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)), tumor growth factor-\(\beta\) (TGF-\(\beta\)), and vascular endothelial growth factor (VEGF) did not have significant difference between groups. TGF-\(\beta\) and VEGF regulate matrix deposition, angiogenesis, and epithelialization in normal wound healing\textsuperscript{[22]} and their levels increase in acute wounds and decrease in chronic wounds\textsuperscript{[21]}. The above findings indicated that presence of biofilm prolonged inflammatory phase of healing and suppressed proliferative activity. As diabetes increases chance of wound infection and biofilm formation, the diabetic mouse model is ideal for studying in vivo effect of biofilms on chronic wound healing in a shorter time span\textsuperscript{[23]}. Although the authors did not observe any systemic effect of biofilm, there was significant weight loss in the test group. In absence of systemic effects, weight loss could be due to poor nutrition, which is an independent factor influencing wound healing\textsuperscript{[24]} and could have confounded the results. Roche et al\textsuperscript{[8]} studied a Meticillin-resistant \textit{S. aureus} (MRSA) biofilm in porcine full-thickness dermal wounds. Pig is a preferred model for wound healing research due to its close resemblance with human skin\textsuperscript{[25]}. They observed significant difference between wound sizes measured at 7 to 14 days of wounding (\(P < 0.0001\)). A high bacterial bioburden (10\(^8\) cfu/g of tissue) was observed at the early part of healing (day 4) which subsided to 10\(^6\) by the 10th day. Similar observation was made by Gurjara et al\textsuperscript{[26]} in their study on rabbit ear wound model (discussed later). Of interest was the observation that irrespective of species of bacteria involved, biofilm inhibit wound healing. Therefore, it may be concluded that it is the host response in form of persistent inflammation which is the prime factor responsible in delaying healing.

Gurjara et al\textsuperscript{[26]} studied the effect of \textit{S. aureus} biofilm on rabbit ear wound model, which closely mimics a chronic wound due to exposed perichondrium. The outcome was compared between 2 groups of wounds containing biofilm and planktonic bacteria, respectively, against a control group. A sharp rise in bacterial population was observed till the fourth day, which dropped to 10\(^6\) cfu/g of tissue, a finding in concordance with that of Roche et al\textsuperscript{[8]}. This was probably due to initial clearance of the planktonic bacteria leaving behind the more stable biofilm population, indicating a steady state the biofilm is thought to maintain with its host\textsuperscript{[7]}. Significant difference in the epithelial and granulation gaps were noted on the 12th day. The large epithelial gaps in the biofilm group were likely due to poor keratinocyte migration. Kintarak et al\textsuperscript{[27]} demonstrated that biofilm staphylococci express a cell surface-related fibronectin binding protein. For keratinocyte migration to happen, fibronectin receptors on keratinocytes need to bind with fibronectin in the matrix\textsuperscript{[28]}. The fibronectin binding protein of the biofilm bacteria can thus directly inhibit epithelialization. Interestingly cartilage necrosis was found in the more acute planktonic group as compared with biofilm group, an observation which strengthens the view that biofilms tend to maintain a steady state interaction with the host. This was also supported by the observation that a significantly low-grade host response, measured through IL-1\(\beta\) and TNF-\(\alpha\), was elicited by the biofilms compared with the active infection by the planktonic form. However, the authors did not find any weight loss in the animals with biofilms as opposed to the diabetic mouse model of Zhao et al\textsuperscript{[19]}.

A number of other animal studies have also confirmed the delay in healing caused by wound biolms\textsuperscript{[9,15,18,29]}. Schaber et al\textsuperscript{[3]} demonstrated a delay in reepithelialization of thermal cutaneous wounds in mouse when infected with \textit{P. aeruginosa} biofilms. A significant observation was the development of \textit{Pseudomonas} microcolonies around capillaries. The authors concluded that these may be the starting point of systemic invasion frequently seen in burn patients. However, according to Wolcott et al\textsuperscript{[7]}, this finding probably suggests proximity of bacteria to a nutrition source as red corpuscles and exudate from blood vessels is a principal source of protein and iron for the bacteria.

The significance of the study by Seth et al\textsuperscript{[9]} lies in the use of a polymicrobial biofilm model containing \textit{Staphylococci} and \textit{Pseudomonas} on rabbit ear wounds. Both species survived until the wounds were harvested on the 12th day. The authors observed a significant upregulation of IL-1\(\beta\) and TNF-\(\alpha\) in the mixed population than in the pure groups. When the wild type Staphylococcal strain was replaced with a mutant one, the cytokine expression decreased. Therefore, it can be argued that inflammatory response to a polymicrobial biofilm is additive in nature and the presence of multiple species is not mutually repressive. Moreover, the epithelial gaps were significantly bigger in the polymicrobial group indicating that effect of biofilm on wound healing is influenced by the polymicrobial nature of resident flora in the wound. However, it needs to be seen whether the same holds true in human wounds.

Effect of biofilm on various phases of wound healing

In vitro models have been a mainstay of biofilm research. They project a pure picture of bacteria and immune cell interaction, but lack the overall effect of a fully formed complex biofilm on an organized immune system. Although biofilms affect all phases of wound healing directly or indirectly, maximum insult occurs in the inflammatory phase\textsuperscript{[7]}. Jensen et al\textsuperscript{[30]} compared the effect of polymorphonuclear cells (PMN) on \textit{P. aeruginosa} biofilm and planktonic bacteria in vitro. PMN-induced killing was measured by chemiluminescence assay. The response of PMN cells was significantly lesser in the biofilm group as compared with the planktonic group, indicating that the PMN cell action decreases in presence of biofilm bacteria. However, they were unable to measure the percentage of biofilm bacteria which actually came in contact with the PMN cells.
Jensen et al.\textsuperscript{[31]} in their in vitro study observed that rhamnolipids, a Q5 molecule produced by \textit{P. aeruginosa}, rapidly necroses PMN cells when incubated with the biofilm supernatant. On the basis of this observation it may be assumed that PMN activity can be inhibited by various biomolecules elaborated by the bacteria and a direct cell to cell interaction does not. Rhamnolipids can also inhibit phagocytosis by acting as a protective shield\textsuperscript{[29]}. McClure and Schiller\textsuperscript{[32]} reported that rhamnolipids caused macrophage membrane distortion and inhibited bacterial binding, phagocytosis, and destruction of phagocytosed bacteria. Not only fewer macrophages were able to bind bacteria, but the number of bacteria cleared by each cell was also decreased. Schooling and Beveridge\textsuperscript{[33]} observed that lipopolysaccharide (LPS) from gram negative bacteria induces chemotaxis and stimulates neutrophils to produce chemokines, but also affects neutrophil function by altering membrane-related phosphatidylserine residues. The above observations confirm that multiple bacterial cell products in biofilms can strongly affect various cellular functions in the inflammatory phase.

Because of altered phenotype and metabolism, the biofilm bacteria are less susceptible to killing by immune cells. Two possible outcomes of a biofilm have been described. If the bacterial virulence is very high, tissue necrosis takes place for deriving nutrition\textsuperscript{[34]}, In the other situation, after a steady-state is reached, low grade virulence factors such as bacterial DNA and cell wall LPS induce chemotaxis, PMN infiltration and upregulation of proinflammatory cytokines\textsuperscript{[35]}. This maintains a state of hyper-inflammation and exudate formation, which provides continued nutrition to the bacteria. At the same time bacterial death is decreased by inhibition of opsonization and trapping which provides continued nutrition to the bacteria. At the same time senescence of keratinocytes\textsuperscript{[44]} was the cause of decreased migration. Loryman and Mansbridge\textsuperscript{[45]} reported significant decrease of keratinocyte migration and increased apoptosis in presence of LPS from biofilm-derived \textit{P. aeruginosa} and \textit{Escherichia coli}. But the migration inhibition was reversed when blocking antibodies to TLR-2 and TLR-4 were used, confirming that the inhibition of migration is mediated through TLR receptors. In a splinted mouse cutaneous ulcer model, Schierle et al.\textsuperscript{[18]} observed significant delay in reepithelialization in presence of Staphylococcal biofilm. The effect was reversed by adding RNAIII inhibitory peptide, a QS inhibitor. Their result confirmed that bacterial Q5 molecule is the cause of delay in epithelial resurfacing. In another article by Marano et al.\textsuperscript{[46]}, the effect of \textit{Staphylococcus} and \textit{Pseudomonas} biofilm broth on cellular toxicity, proliferation and migration of keratinocytes were studied in vitro. The biofilm conditioned broths of both species inhibited cellular proliferation, and both were cytotoxic in higher concentrations. But only \textit{S. aureus} conditioned broth inhibited cellular migration. On proteomic analysis, it was found that the effect of \textit{Staphylococcus} was mediated by a protein, whereas that of \textit{Pseudomonas} was mediated by a small molecule. Moreover, they were able to isolate a number of proteins and enzymes with putative effects on delayed healing. Thus, it seems that not one but a number of bacterial products in biofilm act as inhibitors to keratinocyte proliferation and migration and thus lead to delayed wound resurfacing.

Conclusions

Our knowledge of biofilm and its influence on wound healing, especially chronic wound healing is still evolving. A number of observations generated from experiments in laboratory or animal models have helped to understand the pathogenesis of delayed healing in chronic wounds colonized with biofilm. But, as the biofilm itself is highly complex, individual bacterial model is insufficient to simulate a true complex clinical picture. Lack of human experiments due to ethical reasons provides little knowledge of its effects in chronic wounds. It is believed that the basic pathophysiology of biofilm involves manipulation of host inflammatory response, and turning it to advantage in deriving a continuous source of nutrition. The inability of the host immune system to inhibit hyperinflammation and induce conversion of innate immune response to an adaptive immune response leads to arrest of wound healing in the inflammatory phase, and progression to the proliferative phase and epithelialization. The biofilm thus affects all phases of wound healing directly or indirectly, and is at present considered an independent factor in delaying normal wound healing.

Ethical approval

None.

Sources of funding

None.

Author contribution

B.G.: Literature search. S.M.: Drafted the manuscript. S.B.: Drafted the manuscript and revised it for intellectual content.

Conflict of interest disclosures

The authors declare that they have no financial conflict of interest with regard to the content of this report.
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