Biofilms: do they affect wound healing?

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ABSTRACT

Biofilms are known to exist in wounds, and it is suspected that their presence may delay wound healing, especially in chronic wounds; however, the evidence to support or refute this is not yet conclusive. This literature review has found that there is some evidence, both in vitro and in vivo, that the extracellular polysaccharide (EPS) matrix protects the biofilm from some inflammatory processes key to wound healing. The mechanisms of these effects and how this translates into clinical practice are still unknown. Strategies to manage biofilms within wounds are being investigated and may include use of silver, surgical debridedment, antibiotics and quorum-sensing inhibitors but no firm conclusions can yet be drawn from these studies. In conclusion, while there is a growing body of evidence to suggest that biofilms do indeed influence aspects of wound healing, there is still a large gap in our understanding of how this affects the wounds of clinical patients or how to improve rates of healing.

Key words: Biofilm • Extracellular polysaccharide • Inflammation • Wound healing

INTRODUCTION

It is recognised that in nature bacteria exist not as free floating individuals, planktonic micro-organisms, but as sessile communities attached to a surface (1). Where these communities form permanent attachments and produce an extracellular polysaccharide (EPS) matrix they may be described as biofilms (2). In the human body there are many surfaces upon which biofilms may form including skin, genito-urinary tracts, respiratory epithelium and the gut (3), and it has been suggested that they are involved in up to 80% of all infections (4). Advantages to the bacteria of living in a biofilm include greater protection from host immune response, greater resistance to antibiotic treatment and easier gene transfer leading to sharing of advantageous attributes such as increased virulence (3).

During wound healing biofilms are considered to interfere mainly with the inflammatory process by two mechanisms: first, evasion of the immune response by the EPS matrix and second by induction of a chronic non healing inflammatory phase (5). During the inflammatory phase neutrophils are the first line of cellular defence against bacteria, recognising microbes and engulfing bacteria by phagocytosis (6). Production of proteinases and reactive oxygen species by neutrophils damages surrounding microbes as well as the surrounding tissue (7). The lifespan of a neutrophil is short with apoptosis and engulfment by macrophages signalling a change from early to late inflammation and monocytes becoming the predominant leukocyte present (8). Monocytes, which mature into macrophages, not only continue with bacterial killing but also play a vital role in the activation of the adaptive immune system (9).

The influence of biofilms on wound healing is an area of increasing interest, and much research appears to surround the influence on inflammation. This paper reviews the available literature on the role of biofilms in wound healing with a specific focus on inflammation.

BIOFILMS

The production of EPS matrix appears to be triggered when cells adhere to a surface as showed by Davies *et al*. (10) who compared activity of alginate promoter genes in *Pseudomonas aeruginosa* cultured on a Teflon

Key Points

- biofilms are a community of bacteria living together attached to a surface and can be found in both acute and chronic wounds
- an extracellular polysaccharide matrix gives the biofilm some protection from the inflammatory response, evading phagocytosis and attenuating neutrophil action
- quorum sensing, signalling between bacteria once the biofilm reaches a critical density, may additionally promote virulence within the biofilm
- current evidence for the above points is mainly through in vitro studies and mainly consider only two bacterial species, *Pseudomonas aeruginosa* and *Staphylococcus aureus*, which may not accurately reflect the ecological diversity of biofilms
- there is very little direct in vivo evidence of how a biofilm affects the rate of wound healing in the clinical setting

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mesh or in planktonic form in culture medium. Davies *et al*. (10) indirectly measured an alginate promoter gene as well as directly measuring EPS components to ensure that EPS production was attributable to the promoter gene. Both promoter gene and EPS were increased at least twofold in the mesh-grown biofilm in comparison with planktonic forms (10). While this study is limited in that it considers only one bacterial species on one specific surface it does show the ability of bacteria to alter phenotype in response to the local environment.

The formation of biofilms in vitro appears to be dependent on extracellular signalling between bacteria (11). This effect has also been named quorum sensing (QS) where once a critical bacterial population density is reached (the 'quorum') extracellular signalling occurs and allows the population to change its phenotype (12) to become a biofilm. In order to examine this concept in vivo Schaber *et al*. (13) compared biofilm formation in wild-type *P. aeruginosa* and a strain deficient in QS ability and found that both types were able to form biofilm with no major morphological differences. The QS deficient biofilm was less invasive, however, and this may be attributable to reduced stimulation of virulence factors which appears to be another function of QS molecules. This was in a murine model of an acute thermal injury so also provides evidence that biofilms can form in acute as well as chronic wounds (13). This contrast between the different behaviour of *P. aeruginosa* in vitro and in vivo highlights the difficulties in interpreting evidence, and in the case of QS although one specific isolated QS molecule may prevent biofilm formation in vitro this cannot be extrapolated to the in vivo setting.

Direct evidence of the presence of biofilms in wounds has been provided by James *et al*. (14) where biopsies were taken from 16 acute and 50 chronic wounds and examined by both light and scanning electron microscopy. Thirty of the chronic wounds (60%) in comparison with only one acute wound (6%) showed the presence of biofilm, indicating a lower prevalence in acute wounds; however, there is no description of how long after injury the biopsies were taken which may affect detection rates. The sample of chronic wounds was very heterogenous with no significant difference in the presence of biofilm between different types of wounds (diabetic foot ulcers, pressure ulcers and venous leg ulcers) (14), but no analysis of other potentially contributing factors was performed, for example duration of wound or patient comorbidities. The polymicrobial nature of biofilms was also identified by James *et al*. (14) which is a finding consistent with previous studies which have shown chronic wounds to contain multiple species, most commonly including, *Staphylococcus aureus* (93·5%), *E. faecalis* (71·7%) and *P. aeruginosa* (52·2%) (15). Standard culturing techniques to identify bacteria resident within biofilms in chronic wounds does not appear to be effective with no correlation seen between standard culture results and fluorescence in-situ hybridisation for *S. aureus and P. aeruginosa* (16). New methods for identification of bacterial biofilms deep in the wound tissue are needed to enable better understanding of wound microbiology and more targeted treatment (16).

BIOFILMS IN WOUND HEALING

The function of the EPS in evading the immune response has been a subject of debate with particular interest focussing on the patients with cystic fibrosis (CF) and chronic *P. aeruginosa* infection. The killing of bacteria via phagocytosis requires opsonisation (17) which is the process of labelling of cells for recognition by phagocytes and subsequent engulfment (18). The EPS has been shown to interfere with this process with serum from infected CF patients, containing complement opsonins, able to effectively kill *P. aeruginosa* in planktonic state but not while in a biofilm (17). Meluleni *et al*. (17) further showed that serum with antibodies to EPS was able to kill the bacteria in biofilm significantly more effectively ($P < 0{\cdot}001$). Microscopy showed that in biofilms exposed to serum without EPS antibodies, phagocytes penetrated the biofilm but were surrounded by dense EPS. The authors have suggested that the EPS prevents the detection of opsonins on the bacterial cell wall by phagocytes. This theory is supported as C3, the most important opsonin in complement-mediated immunity (18), was seen successfully attached to bacterial cell walls within the biofilm. Furthermore, in biofilms exposed to serum with EPS antibiodies, C3 was seen attached to the EPS and showed effective bacterial cell clearing (17). This study by Meluleni *et al*. showed both a quantitative effect as well as a potential mechanism, and while this

was an in vitro study the biofilms used showed significant similarity to those taken from lung tissue biopsy (17), suggesting that this is a clinically useful model.

Further investigation into immune cell interaction with biofilms was performed by Leid *et al*. (19) who looked at the ability of leucocytes to adhere to and penetrate a biofilm under conditions of flow or stasis. *S. aureus* biofilms were used and under conditions of flow, as a model for blood flow through a vessel, human leucocytes penetrated the biofilm; however, under static conditions the leucocytes only adhered to the biofilm surface (19). The biofilms used under the two conditions were not directly comparable as the biofilms grown in static conditions were only 2 days old; however, the biofilms grown under flow were 7 days old, and this maturity and change in structure may have also influenced results, potentially allowing easier penetration by leucocytes in the more mature biofilm through nutrient channels (19). Biofilms were also exposed only to the leucocytes for a short period of time (1–2 h), and this may not give an accurate picture of leucocyte action over an extended period of time which is more likely to be the case in vivo. Leid *et al*. (19) also found that leucocytes penetrating the biofilms were unable to engulf bacteria within the structure and were seen to have a 'halo' or a zone clear of bacteria around them. These findings may be consistent with those found by Meluleni *et al*. (17) where a dense layer of EPS was seen around phagocytes within the biofilm. Further investigation with better control of variables, including biofilm maturity, would help to clarify if leucocyte penetration of biofilms was actually seen as a result of flow.

Evading the immune system chemical signals actively evinced by biofilms may also directly compromise certain cell lines. During the development of a biofilm it has been found that $3OC_{12}$-HSL, an extracellular signalling molecule, is produced by *P. aeruginosa* and promotes the development of a complex, structured biofilm (11). Using a synthetically derived source of this signalling molecule, Tateda *et al*. (20) showed that $3OC_{12}$-HSL had cytotoxic activity on murine macrophages and neutrophils. As 50% of cells exposed to $3OC_{12}$-HSL were found to be apoptotic after 4 h, in comparison with 5% of controls, it is proposed that the mechanism of cytotoxicity was via acceleration of apoptosis. This shows active suppression of phagocytes by a *P. aeruginosa* signalling molecule; however, this is in isolation from other chemical signals that would be present in vivo. The authors also state that $3OC_{12}$-HSL has no effect on epithelial cells (20); however, the results are not shown, and this may have important implications on biofilm interference with the proliferative stage of healing.

Despite the apparent ability of biofilms to evade and kill neutrophils, they do appear to maintain some of their normal functions in response to infection. Again using *P. aeruginosa* biofilm, Jesaitis *et al*. (21) found that functions retained by neutrophils were the ability to degranulate and to produce reactive oxygen species; however, both of these responses were attenuated at 50–80% of maximal response. The action of these effects on the biofilm was not assessed in this study. Jesaitis *et al*. (21) also confirmed that neutrophils were found within the biofilm, but that cell morphology showed no signs of cell motility. Therefore, the authors suggested that rather than active migration the neutrophils stimulate bacteria to 'flee' from the neutrophils causing them to sink into the matrix, which is an alternative explanation for the 'halo' described previously. However, in contrast to the study by Leid *et al*. (19), neutrophils retained the ability to phagocytose bacteria in biofilm (21), but as different bacterial species were used in each study the results may not be directly comparable. The actions of neutrophils in comparison with monocytes were also studied by Leid *et al*. (22), and while bacterial killing did occur when exposed to neutrophils (30% bacterial killed) this was much greater when exposed to monocytes (88% bacteria killed) over the same period of time. Monocyte killing was dependent on the presence of interferon-gamma, and the killing effects of either leucocyte were only seen in non EPS producing *P. aeruginosa* biofilms. Where EPS was present no statistically significant bacterial killing was seen (22) confirming that it is the EPS itself which prevents leucocyte killing. Three different strains of *P. aeruginosa* were used including one clinically isolated strain which makes the findings more applicable.

An in vivo study by Davis *et al*. (23) examined neutrophil interaction with biofilms 48 h after inoculating fresh pig wounds with *S. aureus*. The biofilms found showed greater

defence mechanisms than planktonic bacteria with neutrophils unable to penetrate the EPS (23). These results are in concordance with the in vitro study by Leid *et al*. (19) and may suggest that the immaturity of 2-day-old biofilm structure prevents neutrophil invasion. Porcine skin is an excellent model for human wounds (24) and therefore use of this model strengthens the clinical applicability of the study by Davis *et al*. (23); however, it may be useful to extend the time scale over which the biofilms are studied.

Biofilms have been shown to delay re-epithelialisation in a murine model; however, the mechanism of this is unknown. Schierle *et al*. (25) used common skin pathogens, *S. aureus* and *S. epidermidis*, to infect acute murine wounds and interrupted biofilm formation with the use of biofilm inhibitors in some subjects. A statistically significant decrease in epithelialisation was seen where biofilm was present (25). Wounds in non infected controls were created on the same animals, eliminating systemic infection as a confounding variable. The method included splinting of wounds in the mice in order to prevent healing purely by contraction which is the major downfall in the use of a murine model (26).

THERAPEUTIC STRATEGIES

Research into possible treatments of biofilms is still in early stages and one of the first studies examined the effect of silver containing hydrofibre dressings in comparison with non silver containing hydrofibres and a control. This was an in vitro study and using three different bacterial species (*S. aureus, P. aeruginosa and E. cloacae*). Percival *et al*. (27) showed that all bacteria within all biofilms were killed within 48 h of exposure to the silver dressing. Exposure to the non silver hydrofibre appeared to inhibit biofilm growth, but there was no reduction in cell viability. Biofilms used were only 24 h old and this may have made them more vulnerable to the effects of dressings than more mature biofilms (11), and the small scale and in vitro nature of the study makes extrapolation of results into clinical care difficult. One strategy designed to tackle the problem of biofilms in chronic wounds has been developed, called biofilm-based wound care (BBWC), of which the main components in addition to standard care are aggressive debridement and addition of anti-biofilm strategies (28). Several anti-biofilm strategies were used including QS inhibitors, antibiotics, silver and chemicals that interfere with EPS and metabolism, but no description or breakdown of which participants received which treatment is given making it impossible to attribute results to any specific therapy. Analysis of 190 patients with wounds and critical limb ischaemia managed using BBWC showed that 77% of wounds healed over the course of the study (28). As there were no controls, these results were compared with a healing rate of 65% from a previous study (29) and deemed statistically significant; however, differences in the study groups make direct comparison unreliable. In order to ratify the results attributed to the BBWC method performing a controlled trial would be ideal, and in order to determine if BBWC actually directly affects the biofilms in wounds direct identification of biofilms and their progress within the wound would be necessary.

CONCLUSION

Biofilms are the natural form of bacteria conferring advantageous defensive mechanisms on the bacterial population. They are found in both acute and chronic wounds, but there is still some debate as to whether biofilms do delay wound healing, and little research is available that draws a definitive conclusion either way. The majority of studies chose to use *P. aeruginosa* with a few studying *S. aureus* and although these are clinically important organisms, a greater diversity of biofilm models would perhaps better reflect the ecological diversity seen in chronic wounds. The primary research available focusses on the role of EPS which appears to act as a barrier to bacterial phagocytosis and attenuate other killing mechanisms used by neutrophils. Certainly when the EPS is absent from an otherwise comparable biofilm, phagocytes appear to kill bacteria effectively. QS molecules may also have multiple roles, both in the production of biofilm and virulence factors as well as a direct cytotoxic effect on host cells. There is less literature available on the effect of biofilms on the proliferative phase of healing and, as with all research in this area, there appears to be significant conflict between in vitro and in vivo studies reflecting the gap in scientific understanding at present. Methods for management of biofilms in wounds are likewise in early stages, but

strategies are being developed and as awareness of biofilms increases this body of knowledge will grow. In conclusion there is currently a limited amount of research on the role of biofilms in wound healing making it difficult to form any definitive answers with the main question still remaining: do biofilms actually affect wound healing? As understanding builds on the current evidence, may be this question will eventually have an answer.

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