Biofilms and Chronic Wounds: Winning the War in Wounds
Prof. Gregory Schultz, Ph.D.

Overview of topics

- Review the four sequential phases of normal wound healing and recognize the **beneficial** effects of **controlled** inflammation and protease activities
- Understand the link between chronic inflammation caused by **planktonic** and **biofilm** bacteria and **elevated protease activities** that **destroy** proteins that are essential to healing (extracellular matrix, growth factors, receptors)
- Recognize the high **tolerance** of **biofilm** bacteria to most antibiotics, antiseptics and disinfectants
- Integrate "**biofilm based wound care**" into **wound bed preparation (TIME)** by debriding biofilms and preventing planktonic bacterial from reforming biofilm

Sequence of molecular and cellular events in skin wound healing
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Controlled wound inflammation is beneficial

- Inflammatory cells kill planktonic bacteria by phagocytosis and reactive oxygen species
- They also release proteases (MMPs, elastase) that remove denatured ECM components and permit wound healing to proceed. Inflammatory cells are not effective against bacteria in biofilms

Is there a common molecular pathology of chronic wounds?

- Diabetic foot ulcer
- Arterial ulcer
- Pressure ulcer
- Venous ulcer

Hypothesis of chronic wound pathophysiology

- Repeated Tissue Injury, Ischemia and Bacteria - Biofilms
- Prolonged, elevated inflammation
- Imbalanced Proteases & Inhibitors, Reactive Oxygen Species
- Destruction of Essential Proteins (off-target)
- Chronic Non-Healing Wound

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Basic background of bacterial biofilms

• Planktonic bacteria – single, non-attached bacteria
• Biofilm bacteria – a structured community of bacteria cells enclosed in a self-produced exopolymeric matrix that is tightly adherent (sessile) to living or inert surface
• Quorum sensing – process by which bacteria molecules shift growth from planktonic to biofilm phenotypes
• Exopolymeric matrix of biofilms consists of predominately of polysaccharides along with bacterial DNA and proteins that are extremely inflammatory to innate and acquired immune systems
• Biofilms provide a protected mode of growth – evolutionary defense against natural predators: bacterial viruses, amoeba, and microbicides. Also protects against phagocytosis (inflammatory cells), antibodies, natural reactive oxygen species (ROS), antibiotics, antiseptics, and disinfectants
• Persister bacteria are quiescent (not metabolically active) and are not killed by antibiotics that only act on metabolically active bacteria

Confocal laser scanning microscopy (top view) of (A) Planktonic Pseudomonas aeruginosa (B) Biofilm community
(C) Schematic representation of polymicrobial bacterial biofilm formation (side view)

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How does the immunological response to biofilms cause tissue damage and impair healing?

- In Panel A, planktonic bacteria can be cleared by antibodies, phagocytosis, and are susceptible to antibiotics.
- Adherent bacterial cells (Panel B) form biofilms preferentially on inert surfaces or devitalized tissue, and these sessile communities are resistant to antibodies, phagocytosis and antibiotics.
- Neutrophils (Panel C) are attracted to the biofilms, but cannot engulf biofilm.
- Neutrophils still release proteases and reactive oxygen species.
- Phagocytic enzymes (Panel D) damage tissue around the biofilm, and planktonic bacteria are released from the biofilm, causing dissemination and acute infection in neighboring tissue.

Costerton, Stewart, Greenberg, Science 284, 1999

High levels of MMP activity in chronic wounds decrease as wounds heal

Low protease activity in chronic wound fluids of pressure ulcers predicts the rate and extent of healing

Costerton, Stewart, Greenberg, Science 284, 1999

Trengove, Stacey, Macauley, Bennett, Gibson, Bundem, Murphy, Schultz. Wound Rep Reg 7:442-452, 1999

Ladwig, Robson, Liu, Kuhn, Muir, Schultz. Ratios of activated matrix metalloproteinase-9 to tissue inhibitor of matrix metalloproteinase-1 in wound fluids are inversely correlated with healing of pressure ulcers. Wound Repair Reg 10:26-37, 2002

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**MMP-9 activity correlates with wound healing time course**

MMP line  Wound Area Time line

Patient C05 - MMPs rise and the wound stalled, MMPs decrease and wound begins to close

D. Gibson, G. Schultz, unpublished data

**Fibronectin is degraded by chronic wound fluids**

Fibronectin is absent in the base of chronic venous ulcers

Fibronectin reappears in ulcer base during healing

Fibronectin profile in plasma shows a single intact band at 250 kDa. In contrast, fibronectin is degraded to lower molecular weight fragments in venous stasis ulcers and in diabetic ulcers. Wysocki and Grinnell. Lab Invest 63:825, 1990

Fibronectin reappears (stable) as ulcer heals. Fibronectin plays a key role in epidermal cell migration. It is degraded by protease in chronic ulcers, but is stable when inflammation and protease levels decrease. Herrick, Sloan, McGurk, Frewer, McCollum and Ferguson. Am J Pathol 141, 1992

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Biofilms identified in 60% of biopsies of chronic wounds but in only 6% of acute wounds


Question:
Does formation of biofilm colonies in a wound retard healing?

Answer:
YES or NO

Biofilm formation by staphylococcal species delays healing of mouse cutaneous wounds

Scherer et al. Wound Rep Reg 17: 304, 2009

Biofilm formation in Cutaneous Mouse Wounds. (a) Gross appearance of untreated wounds. (g) Gross appearance of wounds colonized by biofilm. (e) Gross appearance of wounded control wounds. (c) Gross appearance of infected control wounds. (b) Gross appearance of infected wounds treated with biofilm. (d) Gross appearance of infected wounds treated with biofilm and antibiotics. (f) Gross appearance of infected wounds treated with biofilm and antibiotics.
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Question:
Why are bacteria in biofilms hard to kill?

Answer:
- Exopolymetric material (EPM) of the biofilm
  - Dense matrix impairs diffusion of large antibodies
  - EPM materials chemically react (neutralize) microbicides
  - Negative charges of polysaccharides and DNA bind cationic molecules like Ag+,
    antibiotics, PHMB+
- Persister bacteria have low metabolic activity
  - Antibiotics only kill metabolically active
- Oxygen diffusion to center of biofilm is limited
  - Promotes growth of anaerobic bacteria
- Synergism between different bacteria
  - MRSA secrete resistance proteins
  - Pseudomonas secrete catalase that destroys H2O2

Hypochlorous acid very slowly penetrates biofilm matrix – reaction-diffusion problem

After 60 minutes of exposure to dilute bleach (Dakin’s solution), many bacteria in this biofilm were dying (green cells), but many cells in the interior of the biofilm were still alive (orange cells).

Biofilms are highly tolerant to antibiotics

Tobramycin rapidly kills planktonic Pseudomonas aeruginosa (●) very effectively, but is not effective against biofilm (●).

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Topical antibiotics effectively kill planktonic bacteria in pig skin wounds but only reduce bacteria in biofilms 2-logs after 48 hours.

Thicker biofilm requires higher concentrations of antibiotic to inhibit growth

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<th>IC† (µg/ml)</th>
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*Highest concentration of azithromycin producing no turbidity after overnight incubation with antibiotic with planktonic cells
†Highest concentration producing no turbidity after overnight incubation with antibiotic with biofilm cells. Turbidity was scored by eye as well as by determining the highest concentration that was within the standard deviation of the optical density measurements of 6–8 replicate untreated controls (no antibiotic)

Metabolic activity of *Pseudomonas aeruginosa* in mature biofilms is limited to the surface layers

- Only fluorescent bacteria are metabolically active
- Only located in outer layers of the biofilm matrix
- Antibiotics only kill metabolically active bacteria

Hal-Stoodley et al., BMC Microbiology 2008, 8:173
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Dissolved oxygen gradients measured in biofilm

Z. Lewandowski, D. De Beer, P. Stoodley

Distribution of aerotolerance of bacterial populations in chronic wounds

Scott Dowd, et al., BioMedCentral Microbiology, 8: 43, 2008

Biofilm based wound care

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**Principles of biofilm based wound care**

1. Frequent debridement of wounds to physically remove biofilm communities
2. Use an effective microbicidal dressing after debridement to prevent reformation of biofilms
3. Alter topical & systemic antimicrobial treatments to prevent emergence of dominant bacteria from polymicrobial populations; utilize bacterial DNA identification techniques
4. Biofilm Based Wound Care is part of Wound Bed Preparation (TIME)

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Extending the TIME concept: what have we learned in the past 10 years?*

Leaper DJ, Schultz G, Carville K, Fletcher J, Swanson T, Drake R.  
Extending the TIME concept: what have we learned in the past 10 years?  
Int Wound J, 2012; 9 (Suppl. 2):1–19

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**What is this filmy wound slough?**  
Mainly fibrin - surrogate biomarker for inflammation

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Question:
How quickly can planktonic bacteria form protective biofilms in wounds after debridement?

Which answer is true?
1. 7 days
2. 5 days
3. 3 days
4. 1 day

Biofilm maturity studies indicate sharp debridement opens a time-dependent therapeutic window

Debridement - biofilm will reform

- No matter how good the debridement, fragments will always remain embedded in the wound
  - Those fragments will reattach quickly, and become metabolically active
  - They will start propagating and coalescing, quorum sensing will then take place
  - Within 24 hours, early biofilm will have formed
  - Within 2-3 days formal, mature biofilm will be present
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Question:
Do all antimicrobial wound dressings effectively kill biofilm colonies grown on pig skin explants?

Answer:
YES or NO

Can dressings disrupt & kill mature biofilms?


24 hr continuous exposure of mature PAO1 biofilm on porcine explants

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Antimicrobial dressing efficacy against mature Pseudomonas aeruginosa biofilm on porcine skin explants
Antimicrobial dressing efficacy against mature Pseudomonas aeruginosa biofilm on porcine skin explants Int Wound J, 2013, doi: 10.1111/iwj.12142

Larval debridement therapy

Before treatment After 24hr treatment


Question:
What effect does NPWT alone or combined with instillation of antimicrobial solutions have on killing biofilm colonies grown on pig skin explants?

Answer:
It depends on the instillation solution used

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**Assessment of NPWT + instillation on biofilms grown on pig skin explants**

- **Methods:**
  - Negative Pressure Wound Therapy (NPWT)
  - Instillation of wound cleansing solutions

**Results:**

1. NPWT alone has minimal effects on reducing mature *P. aeruginosa* biofilms when tested using an in vitro pig skin explant model.
2. Combining NPWT with instillation of some wound cleansing solutions significantly reduces CFUs of *Pseudomonas aeruginosa* biofilms:
   - 1% Povidone iodine kills 99%
   - 0.1% Polyhexamethylene biguanide kills 99.99%
   - 0.05% Chlorhexidine gluconate kills 99.9%

**References:**


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## Summary

1. Biofilms are communities of bacteria encased in a self-produced matrix of polysaccharides, protein and DNA that provides high levels of tolerance to antibiotics, antibiotics and antiseptics.

2. Biofilms are present in a high percentage of chronic wounds and they impair healing by stimulating chronic inflammation, leading to elevated levels of proteases and ROS that degrade proteins that are essential for healing.

3. Topical dressings can reduce biofilm CFUs ~1 to 2 logs except sustained release cadexomer iodine dressings - kills biofilm.

4. NPWT alone has minimal effects on reducing mature biofilms when tested using an in vitro pig skin explant model.

5. NPWT + Instillation of some wound cleansing solutions significantly reduced CFUs of *P. aeruginosa* biofilms compared to NPWT control.


Biofilm Based Wound Care is part of Wound Bed Preparation (TIME).