Bioburden-Biofilms in Inflammation

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Learning Objectives

• 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

• Describe key principles of BIOFILM-BASED WOUND CARE that emphasize DEBRIDING BIOFILMS and PREVENTING REFORMATION OF BIOFILMS as part of the STEP-DOWN approach for effective therapies

Sequence of Molecular and Cellular Events in Skin Wound Healing

Four Phases of Healing
1. Hemostasis
2. Inflammation
3. Repair
4. Remodeling

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.

Respiratory Burst In Neutrophils & Macrophages Produces Reactive Oxygen Species (ROS) That Kill Bacterial & Fungi

In the membranes of neutrophils, NADPH oxidase generates superoxide (O$_2^-$), which spontaneously dismutates to H$_2$O$_2$, and is converted to hypochlorous acid (HOCl) by myeloperoxidase (MPO). These reactive oxygen species (ROS), especially HOCl, participate in the killing of bacteria. The right panels show a bacteria being phagocytized and production of ROS (red color) surrounding the yeast cell.

Question: What happens when the respiratory burst is impaired?

Answer: Severe impairment of host resistance to infection occurs. Clinical condition known as Chronic Granulomatous Disease is due to mutated NADPH oxidase.
**Chronic Granulomatous Disease**

- Characterized by predisposition to bacterial and fungal infections
- Associated with decreased oxygen consumption and defective microbial killing
- Due to defective mutation in components of NADPH oxidase complex
- Reduced levels of superoxide anion (O$_2^-$) which is converted to bactericidal reactive oxidants results in decreased levels of:
  - hydroxyl radical (OH$^-$)
  - hydrogen peroxide (H$_2$O$_2$)
  - peroxynitrite anion (ONOO$^-$)
  - oxyhalides (HOCl, hypochlorous acid)

**Controlled MMPs Are Necessary for Wound Healing**

Debridement, Angiogenesis, Contraction, Epithelial Migration, Remodeling

1. removing denatured matrix
2. degrading capillary basement membrane for angiogenesis
3. contraction of ECM by myofibroblasts
4. migration of epidermal cells
5. remodeling of scar

**MMPs in Normal Wound Healing**

MMPs are essential for normal wound healing, BUT must be:

- At the right places
- At the right times
- At the right amounts

**Major Cytokines Involved in Wound Healing**

<table>
<thead>
<tr>
<th>CYTOKINE</th>
<th>CELL SOURCE</th>
<th>BIOLOGICAL ACTIVITY</th>
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<tbody>
<tr>
<td><strong>PRO-INFLAMMATORY CYTOKINES</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor Necrosis Factor alpha TNF-$\alpha$</td>
<td>macrophages</td>
<td>↑ PMN margination and cytotoxicity ↑ MMP synthesis</td>
</tr>
<tr>
<td>Interleukin-1 IL-1</td>
<td>macrophages, keratinocytes</td>
<td>↑ fibroblast and keratinocyte chemotaxis, ↑ MMP synthesis</td>
</tr>
<tr>
<td>Interleukin-6 IL-6</td>
<td>macrophages, keratinocytes, PMNs</td>
<td>↑ fibroblast proliferation</td>
</tr>
<tr>
<td>Interleukin-8 IL-8</td>
<td>macrophages, fibroblasts</td>
<td>↑ macrophage and PMN chemotaxis ↓ collagen synthesis</td>
</tr>
<tr>
<td>Interferon-$\gamma$ INF-$\gamma$</td>
<td>macrophages, T-lymphocytes</td>
<td>↑ macrophage and PMN chemotaxis ↓ collagen synthesis ↑ MMP synthesis</td>
</tr>
<tr>
<td><strong>ANTI-INFLAMMATORY CYTOKINES</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interleukin-4 IL-4</td>
<td>T-lymphocytes, basophils, mast cells</td>
<td>↓ TNF-$\alpha$, IL-1, IL-6 synthesis ↑ fibroblast proliferation, collagen synthesis</td>
</tr>
<tr>
<td>Interleukin-10 IL-10</td>
<td>T-lymphocytes, macrophages, keratinocytes</td>
<td>↓ TNF-$\alpha$, IL-1, IL-6 synthesis ↓ macrophage and PMN activation</td>
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**Hypothesis Of Chronic Wound Pathophysiology**

Repeated Tissue Injury, Ischemia and Bioburden – Planktonic & Biofilms

↑ TNF-$\alpha$, ↑ IL-1, IL-6

Prolonged, elevated inflammation
↑ neutrophils / macrophages / mast cells

Imbalanced Proteases & Inhibitors
↑ Proteases (MMPs, elastase, plasmin), ↓ inhibitors (TIMPs, α1PI), ↑ ROS

Destruction of Essential Proteins (off-target)
↓ growth factors / receptors, ↓ ECM degradation
↓ cell proliferation, ↓ cell migration,

Acute Wound → Chronic Non-Healing Wound

Is There a Common Molecular Pathology Of Chronic Wounds??

Diabetic foot ulcer
Arterial ulcer
Pressure ulcer
Venous ulcer
Chronic Infection by Medical Biofilms

Biofilms Identified in >80% of Biopsies of Chronic Wounds but in Only 6% of Acute Wounds

Distribution of Species

Wound biofilms are linked to delayed healing

Heterogeneous Distribution Of Bacteria In Chronic Wounds

Mono-species Biofilms Verses Multi-species Infections
**Question:** How do biofilms impair healing of skin wounds?

**Answer:** Biofilms stimulate chronic inflammation by increasing release of proinflammatory cytokines that leads to highly increased levels of proteases and reactive oxygen species that degrade proteins that are essential for healing.

**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 tolerant 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

**Healing of Pressure Ulcers is Predicted by Protease Activity in Wound Fluids**


**MMP-9 Activity Correlates With Wound Healing Time Course**

**Conclusion:** Inflammation in chronic wounds must be reduced to levels that lead to low protease activities that allow wounds to heal.

**Action:** Bacterial levels (both planktonic and biofilm) must be reduced for healing.

**Question:** Why are bacteria in biofilms hard to kill?

**Answer:**
- **Exopolymeric material (EPM) of the biofilm**
- **Dense matrix impairs diffusion of large antibodies**
- **EPM materials chemically react (neutralize) microbicides**
- **Persisters have low metabolic activity**
- **Antibiotics only kill metabolically active bacteria**
- **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 $\text{H}_2\text{O}_2$

**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) Costerton, Sci Am, 2001

**Biofilms are Highly Tolerant to Antibiotics**


**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

Principles of Biofilm Based Wound Care

1. Frequent sharp debridement of wounds to physically remove biofilm communities
2. Use an effective, fast acting microbicidal dressing after debridement to manage residual biofilm bacteria and to prevent reformation of biofilms e.g. Cadexomer Iodine
3. Alter topical & systemic antimicrobial treatments to prevent emergence of dominant bacteria from polymicrobial populations; utilize DNA bacterial identification techniques
4. Step-down treatment should be used to rapidly decrease biofilms and proteases that impair healing


Question: Can you see biofilms on the surface of wound beds?

Answer: YES or NO
Most biofilms are NOT visible on the surface of a wound bed, and much of the biofilm is beneath the surface of the wound bed where it is very inflammatory!

What is This Filmy Wound Slough?
Mainly Fibrin - Surrogate Biomarker for Inflammation

Can you see a biofilm in this wound?

Photo provided by Dr Matthew Malone

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Gauze Debridement of Biofilm Bacteria on Pig Skin Explants

Effect of Wiping Only on Total and Biofilm Bacteria

Effect of Daily Wiping + Plurogel on Total & Biofilm Bacteria

Effects of Non-Contact Ultrasonic Wound Cleansing on Biofilms

Larval Debridement Therapy
Question: How quickly can planktonic bacteria form protective biofilms in wounds after sharp 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

Biofilms from three patients were split into two tubes containing saline (control) or saline with 200 ug/ml gentamicin (treatment). After 24 hours of incubation, total and planktonic bacteria were isolated and CFU after treatment were measured. Total levels of bacteria at 0, 1, 2, and 3 days after initial debridement remained consistently high. However, in two of the three wounds, all bacterial were “planktonic” at 1 and 2 days after debridement (full kill by exposure to gentamicin). But by 3 days post-debridement, all three wounds had re-established substantial levels of biofilm bacteria (10^3–10^5 CFU/gm)


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?


Effects of Antimicrobial Agents on Mature Biofilms on Pig Skin Explants

Step-Down Treatment Strategy for Chronic Wounds
1. Biofilms are communities of bacteria encased in a matrix of polysaccharides, protein and DNA that provides high levels of tolerance to antibodies, 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. Biofilm based wound care emphasizes effective debridement combined with effective topical and systemic treatments that effectively prevent reformation of biofilms.

4. Step-down therapy is based on starting with the therapies that most effectively reduce biofilms, inflammation, and proteases (debridement, biofilm killing agents, protease inhibitors) then shifting to advanced therapies that further reduce proteases and provide biological support for repairing the wound bed (granulation tissue, fibroblasts, and epithelial cell proliferation and migration, collagen synthesis) such as growth factors, collagen dressings, biological membranes, and NPWT.