Comparison of macrophage activation and cytokine expression during normal and diabetic wound healing.

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Abstract
Wound healing is impaired in diabetic patients. This complication is a significant health problem that leads to over 70,000 lower limb amputations per year. Currently, 23.8 million Americans have diabetes and another 54 million have pre-diabetes. Unfortunately, the mechanism for impaired wound healing in diabetics is not understood. Macrophages are important for proper wound healing; they can be activated either through a classical pathway or through an alternative pathway. The type of macrophage activation depends on cytokines present in the local microenvironment. Our goal was to examine the differences in macrophage activation and cytokine expression during normal and diabetic wound healing. We used a murine wound healing model that closely mimics human wound healing. Two 5mm full-thickness wounds were created on the dorsal skin of db/db (diabetic) mice or control mice. These wounds were held open with a silicone splint sutured to the skin around the wound to minimize wound contraction. The wounds were biopsied on days 2, 5 and 7 after surgery; the samples were fixed in formalin for immunohistochemical analysis or snap frozen in liquid nitrogen for RNA isolation. Hematoxylin & eosin staining of wound sections showed an influx of inflammatory cells on day 2 in the control mice. These inflammatory cells stained positively for a marker of neutrophils and for Ym1, a marker of alternatively activated macrophages. This pattern of inflammation was also observed in the diabetic wounds but not until day 5. This is in agreement with the observation that diabetic wound healing is slower than normal wound healing. The staining for neutrophils and Ym1 was absent on day 7 in both the normal and diabetic wounds. PCR array analysis of wound RNA showed a 297-fold increase in the level of CR2 mRNA in the diabetic wound compared to the normal wound on day 5. CR2 is a chemokine receptor expressed on the surface of neutrophils and macrophages. This result correlates with the increased inflammation observed in the diabetic wounds on day 5. These preliminary results point to a delay in the early signals that recruit inflammatory cells to the wound in diabetic mice.

Methods
Murine Wound Healing Model
6–8 week old adult C57BL/6 mice and BKS.Cg-m+/-Leprdb/J (db/db) diabetic mice received a subcutaneous injection of Buprenorphine (0.1ml/10g) and were anesthetized with isoflurane. Two 5mm full-thickness punch biopsy wounds were created on the dorsal skin of each mouse. A ring-shaped silicone splint was sutured to the skin around each wound to minimize wound contraction. Mice were observed daily for general health and body weight measurement. The wounds were harvested on days 2, 5, and 7 after surgery.

Histology & Immunohistochemistry
Skin samples were fixed in 10% neutral buffered formalin for 48-72 hours. They were dehydrated and embedded in paraffin. Paraffin sections were stained with hematoxylin and eosin. Antibodies for PMN and ECF-L (Ym1) were used to phenotypically identify neutrophil granulocytes and alternatively activated macrophages respectively. Bound primary antibodies were detected using a biotinylated secondary antibody, the Vectastain ABC-AP kit, and the Vector red substrate. Sections were counterstained with hematoxylin.

Immunohistochemistry
Sections were counterstained with hematoxylin. Antibody, the biotinylated secondary antibody, the Vectastain ABC-AP kit, and the Vector red substrate. Sections were counterstained with hematoxylin.

Results
Figure 1. Delayed wound healing in diabetic mice compared to wild type mice (A) Wound area in WT (n = 3) and diabetic (n = 4) mice across time. Each time point is the mean wound area percentage of the original wound. (B) Blood glucose levels in normal and db/db mice (C) Representative wounds photographed throughout healing. Wounds on the WT mice were essentially closed by post operative day 12.

Table 1. Preliminary Real-time PCR array data. Many fold change increases and decreases were seen in diabetic mice compared to WT. More samples are needed before conclusions can be made.

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Function</th>
<th>Fold up or down regulation</th>
<th>p value</th>
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<tbody>
<tr>
<td>CCR2</td>
<td>Receptor involved in neutrophil infiltration</td>
<td>297.14</td>
<td>0.005</td>
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<tr>
<td>IL-11</td>
<td>Induce acute phase proteins during inflammation</td>
<td>62.85</td>
<td>0.025</td>
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<td>CCR5</td>
<td>Induce migration and localization</td>
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<tr>
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<tr>
<td>CCR10</td>
<td>Induce in vivo inflammatory activity</td>
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<tr>
<td>CCR11</td>
<td>Induce dendritic cells</td>
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<tr>
<td>CCR12</td>
<td>Induce monocytes and dendritic cells</td>
<td>18.60</td>
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</tr>
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</table>

Figure 3. Neutrophil influx is delayed in diabetic wounds. Wild type wounds showed an influx of neutrophils on day 2. The cells were present in (insert location). These cells were cleared from the wild type wound by days 5 and 7. In contrast, neutrophil influx was not seen in the diabetic wounds until day 5.

Figure 4. Alternatively activated macrophages were present in wild type wounds on days 2 and 5, but they were not observed in the diabetic wounds until day 5. By day 7 there is no staining for macrophages in wild type but they are still present in diabetic. ECF-L (Ym1) was used as a marker to identify alternatively activated macrophages.

Summary
- A clear delay in wound healing was seen in diabetic mice compared to WT.
- Histological comparison showed WT mice had a faster onset and resolution of inflammation, faster granulation tissue formation, and higher amounts of new dermal tissue compared to the diabetic mice.
- Infiltration of neutrophils was delayed in diabetic wounds. Influx was not seen until day 5 in diabetic mice compared to day 2 in WT.
- Diabetic wounds showed a delay in alternatively activated macrophages. These macrophages were not detected in diabetic wounds until day 5 and continued through day 7. In contrast, these macrophages were seen on day 2 and day 5 of WT but were absent by day 7.

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