Wound healing in diabetic ulcers
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Chapter 4

Macrophages in diabetic wounds express the activation marker MRP8/14

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SUMMARY

Patients with diabetes mellitus experience impaired wound healing. An underlying abnormal cellular response may be related to the non healing status of chronic wounds, as important differences exist in the cellular infiltrate of acute healing versus chronic wounds. The ulcer edges of diabetic and venous ulcers are abundantly populated by macrophages, the pivotal cell type in the transition from the inflammation phase towards the next phase of wound repair. However, they seem to be unable to direct the repair process towards the proliferative phase and might have lost their activation markers. The objective of this study was to investigate the expression of the activation marker MRP8/14 on CD68+ macrophages in chronic (> 8 weeks) diabetic wounds compared with chronic venous ulcers and acute wounds.

Punch biopsies were obtained from chronic diabetic and venous ulcer margins and upper leg control skin on day 5, 19 and 4 weeks postwounding (p.w.). Activated macrophages were visualized with immunohistochemical staining. Activated macrophages were detected early in normal wound healing (5-19 days p.w.) where they remain present until 4 weeks p.w. and were mainly localized in the perivascular areas. Expression of MRP8/14 on macrophages was detected through all layers of the dermis in chronic wounds, with particular dense aggregates in perivascular areas. Compared with normal wound healing: 1) significantly larger amounts of CD68+ macrophages were detected in the edge of both chronic diabetic and venous ulcers (p<0.002), 2) a significantly higher number of activated macrophages was present in diabetic ulcers compared with venous ulcers, 54.1 % vs 33.9 % respectively (p≤0.04, 95% confidence interval 0.7%- 39.6%) and compared with day 0 (p<0.0001) but not compared with acute wounds, and 3) in venous ulcers less activated macrophages were present compared with any day in the acute wound healing model, however this was not significant. There was a significant correlation between the age of the patients and the number of activated macrophages (r = -0.74, p=0.049). In conclusion, macrophages within chronic diabetic ulcers appear to maintain an activated phenotype.
INTRODUCTION

Wound healing is defined as a sequence of cellular events leading to wound closure. In acute wounds these events follow a fixed pattern starting with hemostasis, followed by the consecutive phases of inflammation, proliferation, reepithelialization and finally tissue remodelling. Many chronic wounds fail to complete these stages.

The predominant cell types in early wound healing are lymphocytes, granulocytes and macrophages. Macrophages play a crucial regulatory role in the transition between wound inflammation and the next phase of wound repair, granulation tissue formation. Their presence is essential for the initiation and maintenance of wound fibroblast activity. Ross and Benditt made the first observation of the central role of the macrophage when they observed histologically that macrophage infiltration always preceded the onset of fibroplasia. Whereas depletion of granulocytes and lymphocytes has little effect on primary repair, depletion of macrophages has demonstrable deleterious effects. In a subsequent study by Leibovich and Ross, guinea pigs were made monocytopenic by administration of systemic hydrocortisone and local antimonocyte serum which resulted in a significant delay in the histological appearance of fibroblasts and collagen deposition in the wound. Regan et al. reported a significant reduction in wound breaking strength and wound collagen deposition after in vivo macrophage depletion by parenteral administration of macrophage-specific monoclonal antibodies. Furthermore, the addition of macrophages to wounds in old mice accelerated wound repair to a rate that was appropriate for young mice.

Macrophages phagocytose and digest pathologic organisms and tissue debris. In addition, after activation in vivo or in vitro, macrophages produce pro-inflammatory cytokines that attract leukocytes, fibroblasts, smooth muscle cells and endothelial cells to the wound site. Some cytokines, such as IL-1, TNF-α, PDGF, FGF and TGF-β, termed the fibrogenic cytokines, directly trigger the proliferation of fibroblasts and/or stimulate the production of connective tissue.

Macrophages at the site of wound repair consist of two populations, both probably have their origin in the bone marrow. The ‘resident’ tissue macrophage, a minor component, appears to be present continuously, while the other, major component, is recruited from circulating monocytes by migration and chemotaxis. Lately, Moore and coworkers reported in a study on venous ulcers that monocytes appeared to be active perivascular but lost their activation markers while penetrating the wound bed as macrophages. Activated tissue macrophages and a subset of peripheral blood monocytes express the surface antigen MRP (myeloid-related protein) 8/14. This biologically active heterodimer of the calcium-binding proteins MRP-8 and MRP-14 has been described
to cross react with neutrophils and occasionally with endothelial cells and epithelial cells, especially in inflamed skin. Although no definite function has been determined yet, there is evidence that MRP8/14 is involved in cell cycle progression, cell differentiation, cytoskeleton-membrane interactions and phosphorylation events. MRP8/14 positive monocytes, isolated by cell separation techniques, release high amounts of TNF-α and IL-1β in contrast to their MRP8/14 negative counterparts, thus emphasizing their role in inflammation.

Important differences exist between the cellular infiltrate observed in normally healing acute wounds and the infiltrate in chronic diabetic and venous ulcers. The ulcer edges of these chronic ulcers are abundantly populated by macrophages, however they seem to be unable to direct the repair process towards the proliferation phase. Fibroblasts derived from chronic diabetic as well as venous ulcers show impaired proliferation rates. Theoretically, this could be induced by insufficient stimulation by cytokines secreted by other cells. The macrophage, which can be often observed in close proximity to fibroblasts in the wound bed, is considered to be a likely candidate for this regulatory role. As a consequence, reduced macrophage activation or a deficit in the interaction between macrophages and fibroblasts could be a possible explanation for delayed wound healing in diabetic ulcers.

The objective of this study was to investigate the expression of the activation marker MRP8/14 on macrophages present at the margin of chronic diabetic wounds versus controls in order to come to a better understanding of the process of delayed wound healing in diabetes mellitus.
**MATERIALS AND METHODS**

**Clinical profile**

This study was approved by the medical ethical committee of the Academic Medical Center, Amsterdam, The Netherlands. Patients were fully informed about the purpose and consequences of this study, and subsequently gave their informed consent. Ten patients with diabetic ulcers (non-insulin-dependent diabetes mellitus) with a mean age of 69.3 years (SD 11.6 years) admitted to the clinic or visiting the outpatient department of our hospital were included in this study. As a reference we compared the results with those of 6 patients with a venous leg ulcer, with a mean age of 74.3 years (SD 13 years), and an acute wound healing model. A chronic ulcer was defined as existing for 8 weeks or longer. Punch biopsies of 4 mm were taken from the margin of chronic diabetic and venous ulcers with a median ulcer duration of respectively, 6 months [range 2-60] and 4 months [range 2.5-6]. The biopsies were taken in such a manner that the epithelial margin was included in the central part of the biopsy. The epithelial margin can be considered as a reference point because normally the epithelium will grow from here into the wound bed, only if the tissue underneath it has reached a certain level of quality. When biopsies are taken at random from the wound bed large differences can be encountered in the composition of the tissue.

The acute wound healing model was studied in five healthy age matched volunteers (mean age of patients 80 years, SD 10.2 years). Acute wounds were made on the upper leg and wound healing was studied at day 5, 19 and 4 weeks post wounding (p.w.). During the study period the wound area was covered with Opsite (Smith & Nephew, York, UK) to establish a moist wound environment. All punch biopsies were taken under local anaesthesia. The biopsies were snap-frozen and stored at -80 °C until further processing.

**Immunohistochemical staining**

*CD68 double staining with MRP8/14*

Six micrometer cryostat sections were cut and mounted on poly-L-lysine-treated microscope slides. The slides were stored at -20 °C until further staining. Serial sections were fixed in acetone for 10 minutes at room temperature and subjected to inactivation of endogenous peroxidase (0.3% H$_2$O$_2$ and 0.1% sodium azide in Tris buffered saline (TBS)) for 30 minutes at room temperature. Aspecific binding of antibodies was minimized by a 30 minutes preincubation with 10% normal goat serum in TBS. The primary monoclonal mouse antibody
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MRP8/14 was incubated in optimal dilutions (1:100, BMA, Augst, Switzerland) on the sections overnight at 4°C followed by a biotinylated goat anti-mouse antibody (1:200, Dako Ltd, High Wycombe, U.K.) for 30 minutes and thereafter an incubation of 30 minutes with streptABCComplex/Hrp (1:400, Dako Ltd, High Wycombe, U.K.) DIG conjugated anti-CD68 (1:500, Dako Ltd, High Wycombe, U.K.) was then incubated for 60 minutes at room temperature followed by sheep anti-DIG/AP (1:100, Boehringer Mannheim, Mannheim, FRG) for 30 minutes. Alkaline phosphatase activity was detected in blue using fast blue BB and peroxidase activity was detected as an orange red color with 3-amino-9-ethylcarbazole (AEC, Sigma, St Louis, MO). Double staining cells were detected by their purple color. Sections were finally fixed in formaldehyde (4%) and mounted with glycerin-gelatin without counterstaining. Negative controls were stained in the same manner as all sections without incubation with the primary antibody.

Cell enumeration

Cells were counted manually using a microscope eyepiece grid. It was not possible to enumerate the cells with a computerized image analysis system because of the close approximation of stained cell membranes within aggregates particularly in the perivascular regions of the chronic venous and diabetic ulcers. The evaluations were performed by two independent investigators, the comparison of the results revealed very similar assessments of the staining patterns (interobserver correlation coefficient r = 0.92, p≤0.0001, 95% CI [0.46-1.4]).

The number of single or double stained cells of three different serial sections of each wound were counted (blinded) up to a depth of 1 mm. These values of each biopsy specimen were adjusted to 10 mm width. The mean value of each biopsy was used for further analysis.

Statistical evaluation

The student's t-test for independent groups was used for statistical evaluation of differences in percentages of activated macrophages with a correction for unequal variances; p values of < 0.05 (two-tailed) were considered significant. The correlation between ulcer duration and age and the number of activated macrophages was analysed with regression analysis.
Macrophage activation in diabetic ulcers

RESULTS

In normal skin only a few CD68+ macrophages can be detected (Fig. 1A) and some of them are MRP8/14 positive. Five days postwounding an influx of CD68+ macrophages and CD68+/MRP8/14- macrophages is noted (Fig. 1B), mainly perivascular, where they remain present until 4 weeks p.w. (Fig. 1C). High numbers of CD68+ macrophages were identified through all layers of the dermis in chronic venous (Fig. 1D) and diabetic ulcers (Fig. 1E). In venous ulcers in general a low expression of MRP8/14 on macrophages was seen (Fig. 1D), whereas in diabetic ulcers a high number of activated macrophages was noted with particular dense aggregates in the perivascular areas (Fig. 1E,F).

Fig. 2 shows the kinetics of activated and non-activated macrophages in acute and chronic wounds. At day 5 p.w. there is an influx of CD68+ macrophages at the wound site (52% of the cells show expression of MRP8/14), where they remain present until day 18 and 4 weeks p.w. (respectively 45.7% and 45.8% expression of MRP8/14). Compared with normal wound healing, a significantly higher number of CD68+ macrophages was detected in the edge of both chronic diabetic and venous ulcers (p<0.002). In diabetic ulcers a significantly higher percentage of activated macrophages (54% versus 34%) was present compared with venous ulcers (p≤0.04, 95% confidence interval difference between means 0.7% - 39.6%). In venous ulcers less activated macrophages were present compared with any day in the acute wound healing, however this was not significant. In chronic diabetic ulcers a significant higher number of activated macrophages was seen compared to day 0 (p<0.0001), no significant differences in number of activated macrophages were observed compared to day 5, 18 and 4 weeks p.w..

In nine out of ten diabetic ulcers more than 42% of the macrophages were activated, in contrast to venous ulcers where three out of six ulcers showed elevated expression (≥41% of cells positive). The remaining one diabetic ulcer and three venous ulcers demonstrated little expression of MRP8/14 (<27% of cells positive) which is comparable to the expression of MRP8/14 found in normal skin (29%).

The mean differences in activated macrophages between the chronic diabetic ulcers, venous ulcers and acute wounds are displayed in Table 1. The mean difference between diabetic and venous ulcers was significant (p≤0.04). Although differences in percentages exist between venous ulcers and the acute wound healing model these were not significant.
Figure 1. High numbers of activated macrophages in chronic diabetic ulcers. In normal skin a few CD68+ macrophages can be detected (Fig. 1a), some of them are MRP8/14 positive. Five days postwounding an influx of CD68+ macrophages and CD68+ and MRP8/14+ macrophages is noted (Fig. 1b), primarily in a perivascular distribution, where they remain present at the wound site until 4 weeks p.w. (Fig. 1c). High numbers of CD68+ macrophages were identified through all layers of the dermis in chronic venous ulcers (Fig. 1d) and diabetic ulcers (Fig. 1e). In both venous and diabetic ulcers a variability of MRP8/14 expression was observed. In venous ulcers in most cases low expression of MRP8/14 on macrophages was noted (Fig.1d). In contrast, in diabetic ulcers mostly high expression of MRP8/14 was seen with particular dense aggregates in the perivascular areas (Fig. 1e,f). Original magnification 25 x (a-e); 50 x (f).
Figure 2. Macrophages are activated in chronic diabetic wounds as in acute wounds in contrast to those in chronic venous ulcers. Sections of normal skin, acute wounds (day 5, 18, 4 weeks p.w.) and sections from the edge of diabetic and venous ulcers. In normal skin only a few macrophages can be detected and a small percentage (29%) is activated. At day 5 p.w. there is an influx of CD68+ macrophages at the wound site (52% of the cells show expression of MRP8/14), which gradually increases towards day 18 and 4 weeks p.w. (respectively 45.7% and 45.8% show expression of MRP8/14). In chronic diabetic and venous ulcers a significantly higher number of CD68+ macrophages is seen compared with any day in the acute wound healing model (p<0.002). In diabetic ulcers a significantly higher number of CD 68+ and MRP8/14+ macrophages was detected (54%) compared with venous ulcers (34%, p<0.04). In venous ulcers less activated macrophages were present compared with day 5, 18, and 4 weeks p.w., however this was not significant. In chronic diabetic ulcers no significant differences were seen in numbers of MRP8/14/CD68+ macrophages compared with day 5, 18 and 4 weeks p.w., but a significant higher number of activated macrophages was detected compared with day 0 (p<0.00001).
### Table 1. Differences in percentages between activated macrophages in chronic diabetic ulcers, venous ulcers and acute wounds

<table>
<thead>
<tr>
<th>Patients</th>
<th>Mean difference (95% CI)</th>
</tr>
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<tbody>
<tr>
<td>Diabetic ulcer vs venous ulcer</td>
<td>20.2% (0.7% - 39.6%)*</td>
</tr>
<tr>
<td>Diabetic ulcer vs day 0</td>
<td>25.1% (15.5% - 34.8%)*</td>
</tr>
<tr>
<td>Diabetic ulcer vs day 5</td>
<td>2.1% (-12.5% - 16.7%)</td>
</tr>
<tr>
<td>Diabetic ulcer vs day 19</td>
<td>8.4% (-2.7% - 19.5%)</td>
</tr>
<tr>
<td>Diabetic ulcer vs 4 weeks p.w.</td>
<td>8.3% (-2.4% - 18.9%)</td>
</tr>
<tr>
<td>Venous ulcer vs day 0</td>
<td>4.9% (-13.9% - 23.8%)</td>
</tr>
<tr>
<td>Venous ulcer vs day 5</td>
<td>-18.1% (-38.3% - -2.2%)</td>
</tr>
<tr>
<td>Venous ulcer vs day 19</td>
<td>-11.8% (-30.8% - 7.2%)</td>
</tr>
<tr>
<td>Venous ulcer vs 4 weeks p.w.</td>
<td>-11.9% (-30.8% - 6.9%)</td>
</tr>
</tbody>
</table>

* p<0.04)

**Other MRP8/14 positive cells**

There was no correlation between the number of activated macrophages (CD68+/ MRP8/14+) and the number of other MRP8/14+ cells in the sections (r= 0.5, p<0.11).

**Clinical parameters**

Patient age: a correlation was found between age and number of activated macrophages (Fig. 3). For both diabetic and venous ulcer patients, with every increase of 1 year in age, 0.5% less activated macrophages were present in the chronic wounds. A significant correlation between age and number of activated macrophages was found for diabetic ulcer patients r = -0.74, p<0.049, 95% confidence interval [-1.48 - 0.004]).

Ulcer duration: the venous ulcers existed for less than 6 months (mean 4 months, median 4.25 months) and showed less MRP8/14+ macrophages than the more chronic diabetic ulcers (mean 16 months, median 6 months). No significant correlation was observed between the duration of the diabetic and venous ulcers of these patients and the number of activated macrophages.
Fig. 3. Correlation between MRP8/14 and CD68 positive cells and patient age.
Our results clearly show that macrophages in chronic diabetic ulcers are activated in similar levels as in acute wounds. Moore et al. observed a lack of activated macrophages in venous leg ulcers and hypothesized that, due to unknown factors in the wound environment these macrophages lose their activation markers while penetrating the wound bed. This hypothesis does not seem to account for diabetic ulcers. On the contrary, the expression of MRP8/14 by diabetic ulcer macrophages may be interpreted as a normal response to the varying signals they receive from their micro environment as acute wounds express the same pattern. However, since the diabetic ulcers already existed for an average of 16 months, the expression of MRP8/14 may also be interpreted as increased or prolonged macrophage activation. This may be provoked by 'pathological' stimulants such as antigen-antibody complexes, endotoxins or bacterial products. This in turn may increase or prolong fibroplasia, collagen deposition and angiogenesis leading to fibrosis often noticed while sampling biopsy specimen. Our observation in human wound tissue is in concordance with the animal study of Wetzler et al. They also reported elevated numbers of macrophages in the wound tissues of genetically diabetic mice at late stages of wound repair and hypothesized that a sustained expression of macrophage inflammatory protein-2 and macrophage chemoattractant protein-1 might be responsible. We were not able to confirm the earlier mentioned observation of Moore et al. for venous ulcers in our study, however a trend was observed. Although relatively less activated macrophages were detected in venous ulcers compared with acute wounds, this was not significant.

One of the major problems in the investigation of macrophage densities within tissues is the lack of specific pan-macrophage markers, which recognize all macrophages independent of their state of activation, and do not cross-react with other components such as hemopoietic cells, fibroblasts, endothelium etc. In our study the epidermis of most but not all chronic ulcers as well as acute wounds showed positive staining for MRP8/14, which probably signified proliferative activity of the keratinocytes. In other skin diseases with an inflammatory involvement of the epidermis, like LE, lichen planus, bullous pemphigoid, erythema multiforme, psoriasis etc., the same observations were made and could not be ascribed to uptake from the dermis.

There was a significant correlation between the age of the patients and the number of activated macrophages, in older patients less activated wound macrophages were detected \((r = -0.74, p<0.049, 95\% \text{ confidence interval [-1.48 - -0.004]})\). An age-related decline in macrophage-dependent function(s) has been described and might be related to impaired wound healing seen during aging.
Macrophage activation in diabetic ulcers

Our data showed that diabetic ulcer macrophages appeared to be activated in similar levels as in acute wounds. However, it is not certain that the macrophages that express the activation marker MRP8/14 on their cell surface, produce a sufficient amount and the appropriate type of cytokines. The macrophage is the pivotal cell type in the transition from the inflammation phase towards the next phase of wound repair, the proliferation phase. Macrophages therefore secrete fibrogenic cytokines e.g. IL-1 and TNF-α that directly trigger the proliferation of fibroblasts and stimulate the production of connective tissue. The observed impaired diabetic ulcer fibroblast proliferation rates\(^1\)\(^7\) and the diminished collagen deposition reported in several studies,\(^2\)\(^7\)\(^-\)\(^9\) together with the observed close proximity between macrophages and fibroblasts in the wounds\(^3\) might suggest a deficit in the relation between the fibroblast and the macrophage. Further studies should focus on the functional status of the activated macrophages.

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