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Short Communication

Contributions of cell subsets to cytokine production during normal and impaired wound healing

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ABSTRACT

The objective of this study was to determine the relative contributions of different cell subsets to the production of cytokines and growth factors during normal and impaired wound healing. Cells were isolated from wounds of non-diabetic and diabetic mice and separated by magnetic sorting into neutrophils/T cells/B cells (NTB cell subset), monocytes/macrophages (Mo/Mp subset) and non-leukocytic cells including keratinocyte/fibroblast/endothelial cells (KFE subset). On both per cell and total contribution bases, the Mo/Mp subset was the dominant producer of pro-inflammatory cytokines interleukin (IL)-1β, tumor necrosis factor (TNF)-α and IL-6 in both non-diabetic and diabetic mice and was a significant producer of vascular endothelial cell growth factor (VEGF)-A, insulin-like growth factor (IGF)-1 and transforming growth factor (TGF)-β1. The NTB subset was also a significant producer of TNF-α and IL-10 whereas the KFE subset contributed significant amounts of VEGF, IGF-1 and TGF-β1. Sustained production of pro-inflammatory cytokines and impaired production of healing-associated factors were evident in each subset in diabetic mice. These data will be useful for further experimental and modeling studies on the role of cell subsets in wound healing as well as for designing therapeutic strategies for improving healing.

1. Introduction

Skin wound healing involves a series of overlapping events involving hemostasis, inflammation, new tissue formation and remodeling. A number of cell subsets contribute to healing, including keratinocytes, fibroblasts, endothelial cells and inflammatory cells [1–4]. The activity of these cells is regulated by cytokines acting in both autocrine and paracrine fashion to bring about efficient healing. In the setting of diabetes, impaired healing is associated with persistent inflammation, reduced angiogenesis and granulation tissue formation, and impaired closure [5–10]. These defects are associated with persistent production of pro-inflammatory cytokines and reduced release of pro-angiogenic and pro-healing factors.

A number of studies have reported that multiple cellular sources may contribute to cytokine production in wounds. The majority of these studies involve either immunohistochemical assessment of tissue sections or cell culture studies using primary cells or cell lines [1,3,4,11]. However, these methods are not optimal for determining the relative contribution of cell subsets to wound cytokine levels. The objective of this study was to isolate cells directly from wounds and measure cytokine release from these cells ex vivo to establish the relative contributions of different cell subsets to the production of cytokines and growth factors during normal and impaired wound healing.

2. Materials and methods

2.1. Animals

Non-diabetic db/+ and diabetic db/db mice on a C57Bl/6 background were obtained from The Jackson Laboratory (Bar Harbor, ME). Experiments were performed on 12–16 week-old mice. All experimental procedures were approved by the Animal Care Committee at the University of Illinois at Chicago.

2.2. Excisional wounding

Each mouse was anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (5 mg/kg) and its dorsum was shaved and cleaned with betadine and then alcohol swab. Four 8 mm excisional wounds were made on the back of each mouse with a dermal biopsy punch and wounds covered with Tegaderm (3M, Minneapolis, MN) [7–9].

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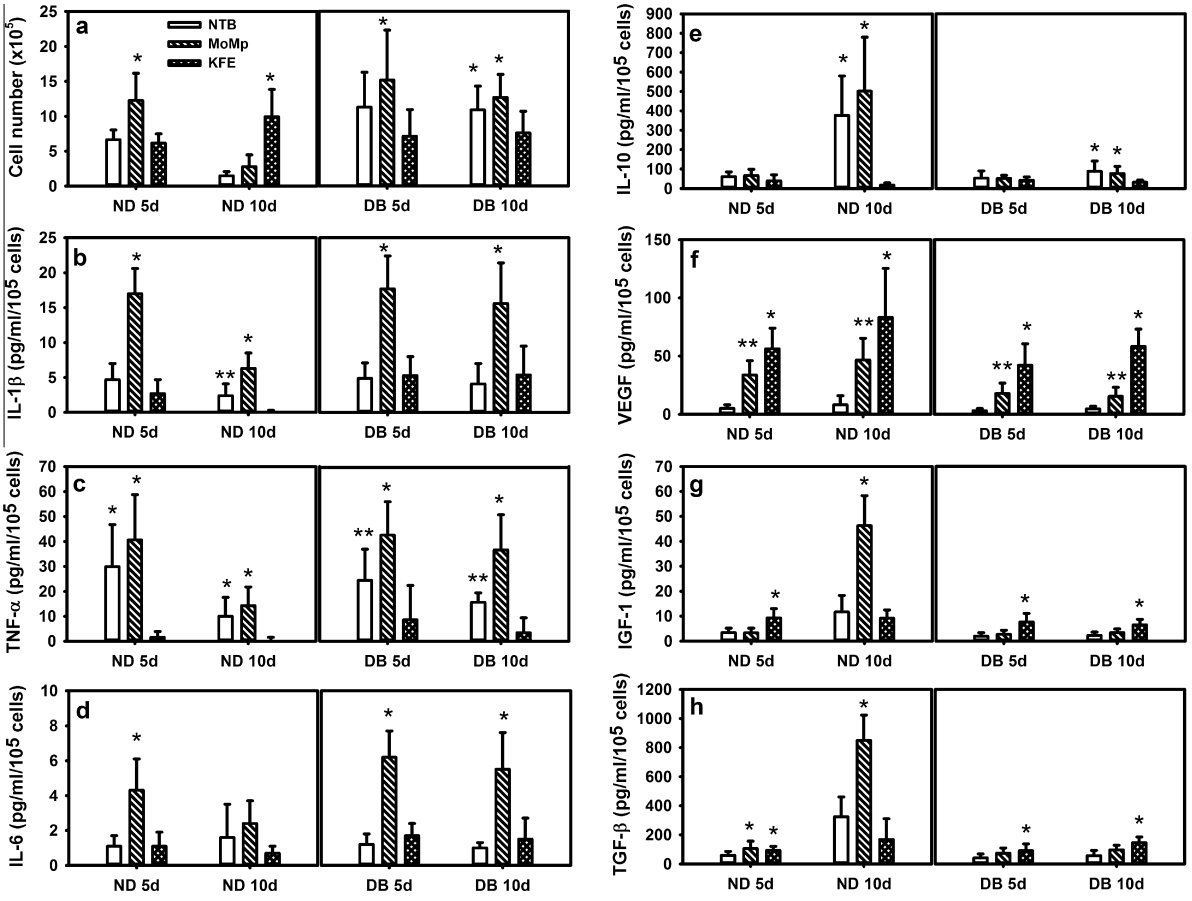

Fig. 1. Cytokine release from cell subsets isolated from wounds of non-diabetic (ND) and diabetic (DB) mice. a: Number of cells in neutrophil/B cell/T cell (NBT) subset, monocyte/macrophage (Mo/Mp) subset and non-leukocytic (KFE) subset on days 5 and 10 post-injury. b–h: Cytokine release per 10^5 cells of each cell subset for b: IL-1β, c: TNF-α, d: IL-6, e: IL-10, f: VEGF-A, g: IGF-1, h: TGF-β1. Bars = mean + SD. *Mean value significantly greater from those for other subsets for same mouse strain and time point. **Mean value significantly less than that for dominant subset but significantly greater than that for remaining subset for same mouse strain and time point. $P < 0.05$, $n = 7$.

2.3. Cell isolation

Cells were dissociated from excisional wounds using an enzymatic digest with collagenase I, collagenase XI and hyaluronidase (Sigma, St Louis, MO) [7–9]. Neutrophils, T cells and B cells (NTB cell subset) were marked with fluorescein isothiocyanate (FITC)-conjugated anti-Ly6G (1A8), anti-CD3 (17A2) and anti-CD19 (6D5) and positively selected using anti-FITC magnetic beads (Miltenyi Biotec, Auburn, CA). Monocytes and macrophages (Mo/Mp cell subset) were then positively selected using anti-CD11b magnetic beads and the remaining non-leukocytic cell subset was likely populated primarily with keratinocytes, fibroblasts and endothelial cells (KFE cell subset). Cell counts were performed using hemacytometer. Flow cytometry was used to verify specificity of the isolation procedure; greater than 90% of the cells in each leukocyte subset stained positively for intended cell markers. Following cell isolation, 5×10^5 cells from each subset were incubated overnight at 37 °C, 5% $C0_2$ in 0.5 ml DMEM supplemented with 10% FBS to assess cytokine release.

2.4. ELISA

IL-1β, TNF-α, IL-6, IL-10 (eBioscience) IGF-1, TGF-β1, and VEGF (R&D Systems) protein levels were measured in cell medium using enzyme-linked immunoassay (ELISA) kits.

2.5. Statistics

Values are reported as means + standard deviation. Wound cell numbers and cytokine release data were compared between cell subsets within each mouse strain and time point using one-way ANOVA. The Student–Newman–Keuls post hoc test was used when ANOVAs demonstrated significance. Differences between groups were considered significant if $P \leqslant 0.05$.

3. Results and discussion

Wound cells were analyzed on days 5 and 10 following excisional wounding, which correspond to the inflammatory and proliferative phases, respectively, in non-diabetic mice. In non-diabetic mice, Mo/Mp were present in the largest numbers on day 5, whereas KFE cells predominated on day 10 (Fig. 1a). In diabetic mice, the NTB and Mo/Mp cell subsets were present at similar levels which were higher than that of the KFE subset on both days 5 and 10, demonstrating the inability to progress through the inflammatory phase in these mice.

For each 10^5 cells, Mo/Mp released the largest amounts of the pro-inflammatory cytokines IL-1β, TNF-α and IL-6 in both non-diabetic and diabetic mice, with the NTB subset also releasing significant TNF-α (Fig. 1b–d). On day 10, when the largest amount of the anti-inflammatory cytokine IL-10 was released, the NTB and Mo/Mp subsets released similar amounts of this cytokine, which was significantly greater than that released by the KFE subset (Fig. 1e). In contrast, the KFE subset released the most VEGF-A in both strains at both time points, with Mo/Mp also releasing a significant amount of this pro-angiogenic growth factor (Fig. 1f). Interestingly, KFE cells released the highest amounts of the pro-healing growth factors IGF-1 and TGF-β1 on day 5 (during the inflammatory phase) but Mo/Mp significantly increased their

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