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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)-1 β , 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)- β 1. 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- β 1. 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.

Keywords

wound healing; diabetes; cytokines; growth factors

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

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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].

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% CO₂ in 0.5 ml DMEM supplemented with 10% FBS to assess cytokine release.

2.8 ELISA

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

2.9 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 $P \leq 0.05$.

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 (Figure 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-1 β , TNF- α and IL-6 in both non-diabetic and diabetic mice, with the NTB subset also releasing significant TNF- α (Figure 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 (Figure 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 (Figure 1f). Interestingly, KFE cells released the highest amounts of the pro-healing growth factors IGF-1 and TGF- β 1 on day 5 (during the inflammatory phase) but Mo/Mp significantly increased their release on day 10 (during the proliferative phase) to become the most prodigious releaser, indicative of the switch in Mo/Mp phenotype at this time point (Figure 1g,h).

We also estimated total cytokine release for each cell subset by multiplying cell numbers obtained for each subset and cytokine release per 10^5 cells from Figure 1. The resulting data demonstrated the importance of both cell number and per cell cytokine release in determining total cytokine release. When analyzed in this manner, Mo/Mp produced the majority of each pro-inflammatory cytokine and the anti-inflammatory cytokine IL-10 in both strains and both time points, with the NTB subset producing less but significant amounts of TNF- α and IL-10. Mo/Mp also produced the majority of VEGF on day 5 with the KFE subset producing the most on day 10. Interestingly, the NTB and Mo/Mp subsets contributed similar amounts of IGF-1 and TGF- β 1 to the total in both strains during the proliferation phase of healing (day 10).

Limitations of this study include potential artifact in cell number data resulting from different efficiencies of isolating different cell subsets from wounds and the potential for artifact in cytokine release data introduced by cell isolation and culture conditions; thus, the cytokine release measurements obtained in this study may not precisely reflect cytokine release in situ during wound healing. However, differences observed in total cytokine release between different stages of healing and between non-diabetic and diabetic mice at least qualitatively reflect levels measured in total wound homogenates reported previously by us and others [1, 3, 4, 7, 11]. Another limitation is that cells were grouped into mixed cell subsets and thus the contributions of individual cell types were not determined within each subset. Other methods for assessing the contributions of cell subsets to cytokine release are limited by lack of quantitative information on relative amounts of cytokine release when performing immunostaining of tissue sections, lack of adequate simulation of the wound environment when performing cell culture studies and potential compensation from other cell subsets when performing in vivo cell depletion studies. Thus, our cytokine release assay overcomes some but not all of the limitations of previous methods for studying the contribution of cell subsets to cytokine and growth factor production during wound healing.

Our data are in agreement with the prevailing view that the Mo/Mp subset dominates production of pro-inflammatory cytokines in wounds and contributes to growth factor production [1–4, 12–14]. Our data are also consistent with previous data from non-diabetic mice showing that Mo/Mp are the dominant expresser of VEGF-A mRNA expression on day 4 post-injury, with non-myeloid cells reaching parity on day 7 and taking over the dominant expresser position by day 14 [15]. In addition, our data indicate that the cellular sources of these pro- and anti-inflammatory cytokines and pro-angiogenic and healing-associated growth factors are similar between non-diabetic and diabetic mice, with persistent release of pro-inflammatory cytokines and impaired release of anti-inflammatory cytokines and growth factors by all subsets, but particularly by Mo/Mp, in the setting of diabetes. Thus, these data are consistent with previous reports indicating that sustained high levels of pro-inflammatory cytokines, including IL-1 β and TNF- α , and reduced growth factor levels, including VEGF, IGF-1 and TGF- β , are associated with impaired healing in diabetes [4, 5, 7, 8, 11].

In summary, the data of the present study provide novel insights into the relative amounts of pro- and anti-inflammatory cytokines and growth factors secreted by different cellular sources during normal and impaired wound healing. These data will be useful for further experimental and modeling studies on the functions of different cell subsets in wounds and for designing therapeutic strategies for improving healing.

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REFERENCES

1. Eming SA, Krieg T, Davidson JM. Inflammation in wound repair: molecular and cellular mechanisms. *J Invest Dermatol.* 2007; 127:514–525. [PubMed: 17299434]

2. Singer AJ, Clark RA. Cutaneous wound healing. *N Engl J Med*. 1999; 341:738–746. [PubMed: 10471461]
3. Kiwanuka E, Junker J, Eriksson E. Harnessing growth factors to influence wound healing. *Clin Plast Surg*. 2012; 39:239–248. [PubMed: 22732373]
4. Werner S, Grose R. Regulation of wound healing by growth factors and cytokines. *Physiol Rev*. 2003; 83:835–870. [PubMed: 12843410]
5. Blakytyn R, Jude E. The molecular biology of chronic wounds and delayed healing in diabetes. *Diabet Med*. 2006; 23:594–608. [PubMed: 16759300]
6. Loots MA, Lamme EN, Zeegelaar J, Mekkes JR, Bos JD, Middelkoop E. Differences in cellular infiltrate and extracellular matrix of chronic diabetic and venous ulcers versus acute wounds. *J Invest Dermatol*. 1998; 111:850–857. [PubMed: 9804349]
7. Mirza R, Koh TJ. Dysregulation of monocyte/macrophage phenotype in wounds of diabetic mice. *Cytokine*. 2011; 56:256–264. [PubMed: 21803601]
8. Mirza RE, Fang MM, Ennis WJ, Koh TJ. Blocking interleukin-1beta induces a healing-associated wound macrophage phenotype and improves healing in type 2 diabetes. *Diabetes*. 2013; 62:2579–2587. [PubMed: 23493576]
9. Mirza RE, Fang MM, Weinheimer-Haus EM, Ennis WJ, Koh TJ. Sustained inflammasome activity in macrophages impairs wound healing in type 2 diabetic humans and mice. *Diabetes*. 2014; 63:1103–1114. [PubMed: 24194505]
10. Jeffcoate WJ, Harding KG. Diabetic foot ulcers. *Lancet*. 2003; 361:1545–1551. [PubMed: 12737879]
11. Barrientos S, Stojadinovic O, Golinko MS, Brem H, Tomic-Canic M. Growth factors and cytokines in wound healing. *Wound Repair Regen*. 2008; 16:585–601. [PubMed: 19128254]
12. Goren I, Allmann N, Yogeve N, Schurmann C, Linke A, Holdener M, Waisman A, Pfeilschifter J, Frank S. A transgenic mouse model of inducible macrophage depletion: effects of diphtheria toxin-driven lysozyme M-specific cell lineage ablation on wound inflammatory, angiogenic, and contractive processes. *Am J Pathol*. 2009; 175:132–147. [PubMed: 19528348]
13. Lucas T, Waisman A, Ranjan R, Roes J, Krieg T, Muller W, Roers A, Eming SA. Differential roles of macrophages in diverse phases of skin repair. *J Immunol*. 2010; 184:3964–3977. [PubMed: 20176743]
14. Mirza R, DiPietro LA, Koh TJ. Selective and specific macrophage ablation is detrimental to wound healing in mice. *Am J Pathol*. 2009; 175:2454–2462. [PubMed: 19850888]
15. Willenborg S, Lucas T, van Loo G, Knipper JA, Krieg T, Haase I, Brachvogel B, Hammerschmidt M, Nagy A, Ferrara N, Pasparakis M, Eming SA. CCR2 recruits an inflammatory macrophage subpopulation critical for angiogenesis in tissue repair. *Blood*. 2012; 120:613–625. [PubMed: 22577176]

Highlights

- Cell subsets were isolated from excisional wounds of diabetic and non-diabetic mice
- Macrophages were the dominant producers of pro- and anti-inflammatory cytokines
- Macrophages were also significant producers of healing-associated factors
- Neutrophils and lymphocytes contributed to pro- and anti-inflammatory cytokines
- Non-leukocytic cells were significant producers of healing-associated factors

Figure 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 (NTB) 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-1 β , C: TNF- α , D: IL-6, E: IL-10, F: VEGF-A, G: IGF-1, H: TGF- β 1. Bars = mean $\pm$ 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. $P < 0.05$, $n = 7$.

Figure 2.

Total cytokine release estimated for each cell subset isolated from wounds of non-diabetic (ND) and diabetic (DB) mice on days 5 and 10 post-injury. Total cytokine release was calculated by multiplying cell numbers obtained for each subset and cytokine release per 10^5 cells in Figure 1. A: IL-1 β , B: TNF- α , C: IL-6, D: IL-10, E: VEGF-A, F: IGF-1, G: TGF- β 1. Bars = mean values for each cell subset, n = 7.