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Restraint stress alters neutrophil and macrophage phenotypes during wound healing

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ABSTRACT

Previous studies reported that stress delays wound healing, impairs bacterial clearance, and elevates the risk for opportunistic infection. Neutrophils and macrophages are responsible for the removal of bacteria present at the wound site. The appropriate recruitment and functions of these cells are necessary for efficient bacterial clearance. In our current study we found that restraint stress induced an excessive recruitment of neutrophils extending the inflammatory phase of healing, and the gene expression of neutrophil attracting chemokines MIP-2 and KC. However, restraint stress did not affect macrophage infiltration. Stress decreased the phagocytic abilities of phagocytic cells *ex vivo*, yet it did not affect superoxide production. The cell surface expression of adhesion molecules CD11b and TLR4 were decreased in peripheral blood monocytes in stressed mice. The phenotype of macrophages present at the wound site was also altered. Gene expression of markers of pro-inflammatory classically activated macrophages, CXCL10 and CCL5, were down-regulated; as were markers associated with wound healing macrophages, CCL22, IGF-1, RELMα; and the regulatory macrophage marker, chemokine CCL1. Restraint stress also induced up-regulation of IL10 gene expression. In summary, our study has shown that restraint stress suppresses the phenotype shift of the macrophage population, as compared to the changes observed during normal wound healing, while the number of macrophages remains constant. We also observed a general suppression of chemokine gene expression. Modulation of the macrophage phenotype could provide a new therapeutic approach in the treatment of wounds under stress conditions in the clinical setting.
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1. Introduction

Wound healing requires the timely orchestration and efficient execution of three major overlapping phases: inflammation, proliferation and resolution/remodeling. These phases prevent bacterial infection, repair the damaged tissue and restore tissue function.

Unfortunately many factors, including stress, can hinder a successful outcome. Stress delays wound healing and impairs bacterial clearance (Padgett et al., 1998; Mercado et al., 2002; Rojas et al., 2002; Horan et al., 2005; Eijkelkamp et al., 2007; Williams et al., 2012). Whether the wound is caused by an accident or a surgical procedure, inefficient removal of bacteria at the site of an injury elevates the risk for opportunistic infection. Infection can potentially prolong discomfort to the patient, increase the cost of wound treatment and extend the hospital stay. Neutrophils and macrophages are innate immune system cells responsible for bacterial clearance at the wound. Previous studies have shown that the appropriate recruitment and functions of these cells are crucial for efficient removal of microbial agents (Bullard et al., 1996; Savill, 1997).

After injury, neutrophils and macrophages leave the peripheral blood to reach the wound. Adhesion molecules on the cell surface (e.g. CD11b) facilitate their migration toward a gradient of chemoattractant leading to the inflammatory site. Neutrophils arrive following a trail of chemokines KC and MIP-2 (Engelhardt et al., 1998; Kernacki et al., 2000; Wetzler et al., 2000). Macrophages recruited by MIP-1α and MCP-1, reach the wound shortly after (DiPietro et al., 1998; Maus et al., 2001). At the wound site, neutrophils and macrophages clear bacteria using oxidative burst and phagocytosis. The phenotype and function of the macrophages vary depending on how they are activated. Pathogen recognition as well as the cytokines and chemokines in the environment shape macrophage activation. Activated macrophages can be classified in three groups: classically activated macrophages (CAM), wound-healing macrophages (WHM) and regulatory macrophages (Edwards

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et al., 2006; Mosser and Edwards, 2008). Each sub-population exhibits specific markers and functions. Classically activated macrophages (CAM) are induced by recognition of microbial patterns via Toll-like receptors (TLR) (Padgett et al., 1998) and cytokines. These pro-inflammatory macrophages express CXCL10 and efficiently kill pathogens (Martinez et al., 2008). Wound-healing macrophages (WHM) arise in response to interleukin-4 (IL-4) and are less proficient than CAM to clear bacteria. However, WHM secrete components of the extracellular matrix and express numerous markers of tissue-remodeling, such as Resistin Like Molecule Alpha (RELMα) and Insulin Growth Factor 1 (IGF-1), which are important during the proliferation and remodeling phases of wound healing. WHM also limit the inflammatory response (Rodero and Khosrotehrani, 2010). Finally, regulatory macrophages also show anti-inflammatory properties, which help in the resolution of inflammation. These cells express CCL1 and IL-10, a potent anti-inflammatory cytokine (Sironi et al., 2006). The macrophage phenotype is not static; macrophages retain their plasticity and are responsive to their environment, which allows them to adapt their phenotype and gene expression profile as wound healing progresses (Daley et al., 2010; Rodero and Khosrotehrani, 2010).

Given the crucial role of neutrophils and macrophages in the removal of bacteria, any factors altering their recruitment and function could impair bacterial clearance. Previous reports have shown that stress modulates neutrophil and macrophage recruitment, chemokine gene expression and the adhesion molecule expression (Curry et al., 2010; Filep et al., 1997; Heasman et al., 2003; Mizobe et al., 1997; Viswanathan and Dhabhar, 2005; Zhang et al., 1998). In addition, stress was reported to alter neutrophil and macrophage microbicidal functions (Ehrchen et al., 2007; Khanfer et al., 2010; Palermo-Neto et al., 2003). Previous studies in a murine model of cutaneous healing showed that restraint stress increased susceptibility to opportunistic infection. Rojas et al. (2002), reported that even though bacterial levels were similar at the time of wounding and 6 h post-wounding, under stress, bacterial clearance was impaired leading to increased bacterial load as early as day 1 after wounding, and up to a 3-log increase in bacterial counts at day 5 post-wounding.

We hypothesized that restraint stress alters recruitment and/or functions of neutrophils and macrophages during wound healing, thereby impairing bacterial clearance. The effect of restraint stress on neutrophil and macrophage recruitment and chemokine gene expression during wound healing was investigated. Anti-microbial functions of neutrophils and macrophages were assessed. The expression of cell surface markers that are involved in adhesion and bacterial recognition by macrophages was also examined. Finally, we explored how restraint stress altered the subpopulations of activated macrophages during wound healing.

2. Methods

2.1. Animals

For all animal experiments in this study, we selected the SKH-1e mouse strain, which are hairless. This mouse has been used widely in wound healing models, dermal research/photosensitivity studies, and safety and efficacy testing. Hairless mice are more susceptible to wounds from fighting because of their relative lack of fur. Female mice are less aggressive and are less likely to develop non-experimental wounds inflicted by littermates during establishment of the litter social hierarchy. Therefore we selected female SKH-1e mouse for the study. In this study, eight-week old female SKH-1e mice were obtained from Charles Rivers, Inc. (Wilmington, MA). Mice were housed in conventional cages, five animals per cage, under a 12:12 light:dark cycle (starting at 18:00), before and throughout the experiments. Water and food were available ad libitum. Animals were allowed 1–2 weeks to acclimate to the cages 7–10 days before the start of the experiment. Animals were handled according to a protocol approved by the Institutional Animal Care and Use Committee.

2.2. Restraint stress

Restraint stress paradigm was used to induce stress in randomly assigned mouse groups. This model provides a consistent physiological and psychological stress response (Sheridan et al., 1991; Zhang et al., 1998; Padgett et al., 1998; Rojas et al., 2002). Each mouse subjected to restraint was placed in a well-ventilated 50 mL conical tube for 12 h/cycle during the active phase. Mice were restrained for three cycles prior to wounding, and five additional cycles after wounding as previously described by Williams et al. (2012), Gajendrareddy et al. (2005). The restraint tubes were cleaned and sterilized between each restraint cycles. Since animals in the tubes did not have access to food and water, control mice were deprived of food and water during the same 12 h periods but were allowed to roam free. As it is well established that restraint stress induces a delay in wound closure (Padgett et al., 1998; Horan et al., 2005; Eijkelkamp et al., 2007), each wound was photographed everyday for each animal, beginning on the day of wounding and analyzed by photoplanimetry. The wound size was determined in order confirm the delay in wound closure in the stressed group, for each experiment (data not shown).

2.3. Wounding and tissue harvest

Mice were anesthetized with 250 μL doses of ketamine-xylazine-saline soluttion (ratio 4:1:35) consisting of ketamine 100 mg/kg and xylazine 5 mg/kg, administrated intra-peritoneally. The dorsal skin was cleaned with isopropanol pads and two full-thickness wounds were created below the shoulder blades using a sterile 3.5 mm biopsy punch (Miltex Inc., York, PA). Mice were anesthetized and the wounds were harvested one and five days post-wounding with a 6 mm biopsy punch (Miltex Inc., York, PA) before the mice were euthanized.

2.4. Measurement of bacteria load in the wound

Bacterial load at the wound was assessed by methods adapted from Rojas et al. (2002). To harvest wounds for bacterial assays, mice were anesthetized (as described above) and wounds were harvested 5 days post-wounding via 6 mm punch biopsy, and homogenized in 1 mL of sterie 1×PBS, using a Tissue-Tearor (Cole-Parmer, Vernon Hills, IL). Serial dilutions (1:10) were plated, in duplicate, on brain–heart-infusion agar (Becton–Dickinson), incubated for 24 h at 37 °C, and quantified by counting the number of colonies formed.

2.5. Myeloperoxidase (MPO) assay

As described by Zhou et al. (1996), harvested wounds were homogenized in 1 mL of 50 mM sodium phosphate buffer, pH 6.0, with 0.5% HTAB (Sigma–Aldrich, St. Louis, MO). Homogenates were centrifuged at 12,000*g* for 20 min and underwent 3 cycles of freeze/thaw for MPO extraction. Supernatants were mixed 1:15 with 80 mM sodium phosphate buffer pH 5.4, containing 16 mM 3,5,3′,5′-tetramethylbenzidine (TMB) (Sigma) previously dissolved in dimethylformamide (Sigma–Aldrich, St. Louis, MO). Reactions were started by adding 0.03% hydrogen peroxide, incubated for 2 min at 37 °C, and stopped by adding 200 mM sodium phosphate buffer pH 3.0. Absorbance was measured at 650 nm for each sample. Units of MPO per wound were determined by regression

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