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Short Communication

Stress-related changes to immune cells in the skin prior to wounding may impair subsequent healing

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ABSTRACT

Higher psychological stress is associated with slower dermal wound healing, but the immunological mechanisms behind this effect are only partially understood. This paper aims to investigate whether immune cells present in the skin prior to wounding can affect subsequent healing in high-stress and low-stress participants. Two studies are presented in which skin biopsies were analysed using immunohistochemistry for numbers of macrophages and Langerhans cells, and immune cell activation (Study 2 only). Immune cells were related to perceived stress levels and subsequent healing. Study 1 included 19 healthy older adults and showed that higher stress was associated with significantly fewer macrophages in the skin. Study 2 included 22 younger adults and showed that higher stress was associated with significantly lower activation of immune cells in the skin. Furthermore, lower activation of immune cells (as measured by human leukocyte antigen (HLA expression)) and fewer Langerhans cells were associated with slower healing. Together these studies show the first preliminary evidence that the number and activation of immune cells in the skin prior to wounding are affected by stress and can impact healing. Larger studies are needed to confirm these effects.

1. Introduction

Psychological stress can slow wound healing (Walburn et al., 2009), however the pathways through which this occurs are not completely understood. Research has shown that people with higher stress have poorer initial inflammatory responses to wounds, including lower pro-inflammatory cytokines and an impaired neutrophil transcriptome (Glaser et al., 1999; Roy et al., 2005). These individuals may have prolonged inflammation later on, as shown in animal models (Mercado et al., 2002). Epinephrine has been shown to prolong inflammation via persistent neutrophil trafficking to the wound (Kim et al., 2014). However no studies have looked at how stress affects the cells present in the skin prior to wounding and whether this can affect subsequent healing.

Immunological constituents of skin tissue include Langerhans cells and resident dermal macrophages. Upon tissue injury, these cells initiate the inflammatory response by recruiting leukocytes, neutrophils, and monocytes, which are important for wound repair (Davies et al., 2013). Moreover, monocytes and lymphocytes play a pivotal role in minimizing infection. Monocytes effectively present and process antigen through expression of class II major histocompatibility complex (MHC) human leukocyte antigen (HLA), activating lymphocytes to mount a swift immune response to infection (Goldsby et al., 2003).

Stress impacts both innate and adaptive immunity in the skin (Hall et al., 2012), with chronic stress shown to suppress skin immune responses (Segerstrom and Miller, 2004). Stress has been shown to affect the proliferation, cytokine secretion, and trafficking of macrophages (Padgett and Glaser, 2003) to reduce Langerhans cell frequencies in the epidermis (Hosoi et al., 1998; Kleyn et al.,

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2008), and to increase susceptibility to wound infections (Rojas et al., 2002).

This paper describes two studies investigating whether skin immune cell characteristics (quantities and activation levels) differ between individuals reporting high or low perceived stress, and whether these differences affect subsequent wound healing speed.

It was hypothesised that people reporting high perceived stress would have fewer immune cells and less immune cell activation in their skin tissue than people reporting low stress levels. It was also hypothesised that fewer immune cells present in the skin would be associated with slower wound healing.

2. Materials and methods

2.1. Study 1

Participants were 19 healthy, non-smoking, older adults in New Zealand (10 males, 9 females; mean age = 78 years, SD = 8 years) from the control condition of a randomized controlled trial investigating the effects of expressive writing on dermal wound healing (Koschwanez et al., 2013). The control condition wrote descriptively for 20 min a day, for three consecutive days about how they spend their time (Time Management). Two weeks post-writing, participants completed a 10-item perceived stress scale (PSS-10) (Cohen et al., 1983) to assess stress over the past week. Participants then received a standard 4 mm diameter punch biopsy wound on their inner, upper, non-dominant arm.

Immunohistochemical staining was performed on the formalin-fixed paraffin-embedded biopsied skin samples using CD1a antibody (Novocastra Liquid Concentrate (NLC)-CD1a-235) to identify Langerhans cells, CD45 (NCL-Leukocyte Common Antigen (LCA)) to identify leukocytes, and CD68 (NCL-CD68) to identify macrophages (Leica Biosystems, Nussloch, Germany) and the Novolink Min Polymer Detection System (RE7290-K; Leica Microsystems, Wetzlar, Germany) as per the manufacturer's instructions. Tissue sections were 5 μm thick.

Digital images (using the 40x objective) of the stained tissue were captured using a Nikon Digital Sight DS-U1 (Nikon Corp., Tokyo, Japan) mounted on a Leica DMR fluorescence microscope (Leica Microsystems, Wetzlar, Germany) using Eclipsenet software (Nikon Corp., Tokyo, Japan). Three representative images were captured per stain, per skin sample. Images were imported into Adobe Photoshop (Adobe Systems Inc. San Jose, CA), where a total of nine 50 μm × 50 μm sections were cropped from the three representative images for further analysis.

To quantify Langerhans cells and leukocytes (CD1a and CD45 expressing cells, respectively), ImageJ 1.46r software (Rasband, 2014) was employed. Briefly, the percent area of positively stained cells was computed for each 50 μm × 50 μm section using digital image analysis, and then averaged across the nine sections. To quantify macrophages (CD68 expressing cells), positively stained structures were counted for each 50 μm × 50 μm section, and then averaged across the nine sections. Cells were counted by two researchers independently, blinded to perceived stress scores and speed of wound healing. A senior histologist was consulted when differences in cell counts could not be reconciled between the two researchers.

Participants were categorized as 'high' or 'low' stress based on the median split of the perceived stress score (on day of wounding). The cut-off score for the median split was 10, with scores of 10 and below being 'low' stress and scores of 11 and above being 'high' stress. Total possible score range was 0 to 40. PSS scores in the 'low' stress group ranged from 1 to 10, mean 4.9 (SD = 3.5), while PSS scores in the 'high' stress group ranged from 11 to 24, mean 15.7 (SD = 4.4). Speed of wound healing was assessed from routine digital images of the wound surface taken every 3–4 days over 21 days. A dermatologist, blinded to participant stress scores and time duration since wounding, rated each wound image as 'healed' or 'not healed': healed was defined as wound closure (complete re-epithelialization with associated wound contracture).

Demographics, immune cell quantities, and wound healing speeds were compared between the two stress groups using Pearson's Chi-square tests, independent *t*-tests, and Mann–Whitney *U*-tests; associations between healing speed and number (or percent area) of immune skin cells were assessed using Spearman correlations. All statistics were performed using IBM SPSS Statistics 20.

Approval was obtained from The University of Auckland Human Participants Ethics Committee. This study was registered at the Australian New Zealand Clinical Trials Registry (trial number 343095; http://www.ANZCTR.org.au/).

2.2. Study 2

The sample was constructed from data collected in two previous wound healing studies involving a total of 60 young, healthy, non-smoking males in the United Kingdom: 24 participants (mean 29 years, SD = 11 years) from the Ebrecht and colleagues (2004) study and 36 participants (mean 22 years, SD = 4 years) from the Scott (2002) study. Exclusion criteria included having a chronic inflammatory condition, taking immunosuppressants, anti-histamines, and high doses of glucocorticoids.

For both these previous studies (Ebrecht et al., 2004; Scott, 2002) , biopsy wounds were created in the inner, upper, non-dominant arm using a standard 4 mm diameter punch. Biopsied skin was collected for analysis. At 7, 14, and 21 days after punch biopsy, wounds were scanned using high resolution ultrasound scanner (EPISCAN HRU Scanner, Longport Intl. Ltd., Silchester, UK). Overall wound healing rate was calculated as the change in wound diameter between Days 7 and 21 post-biopsy, measured from the base of the wound. Wound healing rates between Day 7 and 14, as well as Day 14 and 21, were also calculated.

As a preliminary investigation on whether immune cells present in the skin prior to wounding affect healing in high-stress and low-stress participants, 22 participants were selected from the larger pool of 60 participants. These 22 participants fell unequivocally into two contrasting groups: 11 participants with the lowest perceived stress scores and fastest wound healing rates (mean PSS = 11.45, SD = 1.92, range 9–14) and 11 participants with the highest perceived stress scores and slowest wound healing rates (mean PSS = 29.36, SD = 1.31, range 28–32). Perceived stress scores (PSS-14; (Cohen et al., 1983)) were measured at 14-days post biopsy to assess stress over the past month.

Immunohistochemical staining was performed on the formalin-fixed paraffin-embedded biopsied skin samples using CD1a antibody (clone O1O) to identify Langerhans cells, CD45 (clone F10–89-4) to identify leukocytes, CD68 (clone S14 H12) to identify macrophages, and human leukocyte antigen HLA(clone WR18) to establish level of immune cell activation from AbD Serotec (Bio-Rad Laboratories, Inc., Hercules, CA). Antibody localization was performed using a peroxidase reaction with 3,3′-diaminobenzidine (DAB) tetrahydrochloride (Sigma Aldrich, St. Louis, MO) as the chromogen. Tissue sections were 5 μm thick.

Three to four digital images (using the x20 objective) per stain per participant were captured using a Colour Coolview camera (Photonoc Science, Robertsbridge, E Sussex) mounted on a Leica DMRB light microscope (Leica Microsystems, Wetzlar, Germany). Images were analysed using Image-Pro Plus (Version 3.0.1) (Media Cybernetics, Rockville, MD) to estimate the percent area coverage of Langerhans cells and macrophages. Percent area of

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