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Short Communication

Identification of mRNA of the Inflammation-associated Proteins CXCL8, CXCR2, CXCL10, CXCR3, and β-Arrestin-2 in Equine Wounded Cutaneous Tissue: a Preliminary Study

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ABSTRACT

Horses often sustain cutaneous wounds and healing can be prolonged and difficult to treat. Compared to body wounds, limb wounds heal slower and are more likely to develop exuberant granulation tissue. Differences in healing rates and exuberant granulation tissue formation is attributed to abnormal cytokine profiles. CXCL8 and its receptor CXCR2 are involved in acute inflammation whereas CXCL10 and its receptor CXCR3 are involved in inflammation resolution. β- arrestin-2 regulates inflammation through internalization of G-protein coupled receptors (GPCRs) including CXCR2 and CXCR3. Gene expression of these five inflammation associated proteins have not been previously identified in equine cutaneous tissue and may play a role in dysregulation of inflammation in equine limb wounds. The mRNA expression levels were measured using QuantiGene Plex Assay from cutaneous biopsies collected from surgically created wounds on the limb and thorax on days 0, 1, 2, 7, 14, and 33 from two horses. The mRNA expression levels were measured in mean fluorescent intensity and graphed. We were successful in identifying all five proteins for the first time in equine cutaneous tissue. Preliminary results suggest that there are different expression patterns for CXCL8, CXCR2 and β-arrestin-2 between the limb and thorax but not for CXCL10 and CXCR3. Differential regulation of CXCL8, CXCR2 and β-arrestin-2 may further explain why limb wounds heal differently than body wounds and warrants further investigation.

1. Introduction

Cutaneous wounds are common in horses and often lead to prolonged and complicated healing. Wound healing is a complex process finely orchestrated by cytokines and growth factors that influence all phases of healing and deviations in the amount or order of cytokine release contributes to abnormal healing [1]. Limb wounds heal slower and have a propensity to develop exuberant granulation tissue (EGT) compared with body wounds [2–6]. Past studies have identified that chronic low-grade inflammation in limb wounds causes dysregulation of cytokine profiles, promoting EGT formation [2–9]. Similarly, humans also develop hypertrophic scars and keloids when cytokine release is dysregulated [10–12]. The development of fibroproliferative disorders in both horses and humans can severely affect quality of life and is a major financial burden [10]. Exuberant granulation tissue formation on the limbs of horses shares similar qualities with fibroproliferative disorders in

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people, including a similar contraction to epithelialization ratio, heritability of the disorder, dysregulated cytokine profiles, and haphazard collagen deposition [10,13]. Interestingly, horses and humans are the only species that spontaneously develop these fibroproliferative disorders; thus, horses may be good models for studying aberrant wound healing in humans [10]. Consequently, recording cytokine profiles during wound healing in the horse is important to identify possible time points for intervention to avoid aberrant healing and for establishing its usefulness as a model of wound healing in humans.

Chemokines are small proteins that initiate chemotaxis of leukocytes to the site of inflammation through binding of G-protein–coupled receptors (GPCRs). Chemokines are classified based on their conserved cysteine (C) residues of which there are four groups: CXC, CC, C, and CX3C. The CXC group is further divided into subtypes according to the glutamate-leucine-arginine (ELR) motif located immediately adjacent to the CXC motif. Glutamate-leucine-arginine–positive CXC chemokines are strong neutrophil attractants and promote angiogenesis, and are likewise considered proinflammatory mediators. By contrast, ELR-negative CXC chemokines are poor neutrophil activators but strong lymphocyte and monocyte attractants and are angiostatic; hence, they are considered inflammation-resolving mediators [14,15].

CXC ligand 8 (CXCL8) is an ELR-positive CXC chemokine that is released by damaged cells at the site of injury. As such, it is a potent chemoattractant for neutrophils and triggers angiogenesis. The GPCR for CXCL8 is CXC receptor 2 (CXCR2) and is primarily expressed on the surface of neutrophils and endothelial cells [14,15]. The CXCL8/CXCR2 axis is associated with acute inflammation, and human wound-healing models have identified a direct temporal and spatial relationship between expression of CXCL8 and infiltration of neutrophils into the wound [16,17]. The expression of CXCR2 is tightly regulated to prevent excessive inflammation. Within 5 minutes of binding with CXCL8, CXCR2 internalizes within neutrophils and then is later recycled to the surface [18]. This process is regulated primarily through β-arrestin-2 (βarr2), a ubiquitously expressed intracellular protein that negatively regulates inflammation through internalization of GPCRs, including CXCR2 [19]. βarr2 null mice have improved wound healing secondary to increased expression of CXCR2 by neutrophils that allows continuous binding with CXCL8 and therefore more pronounced neutrophil chemotaxis to the wound, initiating the inflammatory cascade [20]. However, βarr2 null mice also have accelerated tumor growth through continued CXCL8/CXCR2 binding on endothelial cells, promoting angiogenesis [21]. This emphasizes the importance of tight regulation of cytokine production.

In contrast to the effects of CXCL8, CXC ligand 10 (CXCL10) is an ELR-negative CXC chemokine and is released during the proliferative and maturation phases of wound healing. It is angiostatic and it attracts lymphocytes and monocytes, which are involved in resolving inflammation and promoting scar formation [14,22]. The GPCR for CXCL10 is CXC receptor 3 (CXCR3) that is expressed on the surface of T-lymphocytes, monocytes, fibroblasts, endothelial cells, and keratinocytes [22].

The importance of the resolution phase of wound healing is well demonstrated in CXCR3 null murine models [23]. These mice have an adequate acute inflammatory response but delayed re-epithelialization and hyperkeratotic scar formation characterized by haphazard fibroplasia and decreased scar strength. Similar to the relationship of CXCL8 and CXCR2, the CXCL10/CXCR3 axis is also tightly regulated although βarr2 plays a less pivotal role. This tight regulation is especially important to prevent excessive inflammation secondary to T-lymphocyte activation—leukocytes that are also involved in autoimmune diseases. Shortly after ligand binding, CXCR3 becomes internalized by βarr2, but then is degraded rather than recycled as with CXCR2. More CXCR3 must then be translated from mRNA, delaying expression of CXCR3 and preventing further T-lymphocyte activation. In addition to βarr2-mediated internalization, the responsiveness of CXCR3 can also be decreased through binding of intracellular regulatory $G\alpha_{i2}$ subunits to CXCR3, another checkpoint to prevent excessive inflammation by T-lymphocytes [24].

In normal circumstances, the local cytokine environment triggers appropriate phases of acute inflammation, inflammation resolution, and maturation of the wound. However, if appropriate cytokine release is interrupted at any of these phases, delayed healing and dystrophic scarring can occur [1].

To the authors' knowledge, gene expression of these inflammation-associated proteins has not been identified in equine cutaneous tissue. Identification of these proteins is an important first step to understanding the roles they play in equine wound healing. Variations in temporal profiles may further explain why limb wounds heal differently than body wounds in horses. In addition, documentation of temporal cytokine patterns is important to add to our understanding of equine wound healing and strengthens the usefulness of the horse as an animal model for studying fibroproliferative disorders in humans. Furthermore, understanding the role of these proteins in wound healing may lead to novel ancillary treatments including CXC chemokine/GPCR axis manipulation [25,26].

Our primary objective was to determine if mRNA of CXCL8, CXCR2, CXCL10, CXCR3, and βarr2 can be detected in convenience-sampled frozen equine wounded cutaneous tissue at six different time points throughout wound healing using multiplex mRNA assay technology. Our secondary objective was to graph the mean fluorescent intensity (MFI) of the mRNA of these proteins over time for limb and thoracic wounds to determine if there are temporal or site variations to guide future investigations.

2. Materials and Methods

The mRNA expression levels of CXCL8, CXCR2, CXCL10, CXCR3, and βarr2 were measured using the QuantiGene Plex Assay method (Thermo Fisher Scientific, Santa Clara, CA, USA) on frozen equine tissue previously collected from another cutaneous wound healing study. Use of animals in the project was approved by University Animal Care Committee and Animal Research Ethics Board of the University of Saskatchewan. In that study, seven standardized surgical excisional cutaneous wounds were created under general anesthesia on the left lateral metacarpus and left thorax of two 7-year-old Thoroughbred mares on day 0. Resected skin was retained for reference as normal nontraumatized tissue. Both horses received 1.02×10^8 equine umbilical cord blood multipotent mesenchymal stromal cells (MSCs) (eQcell Therapies Inc, King City, Ontario, Canada) injected into the left jugular vein 12 hours after wound creation. Biopsies were collected from the wounds sequentially starting from the most distal and ventral site on the metacarpus and thorax, respectively, on days 1, 2, 7, 14, and 33. All biopsy tissue was immediately snap-frozen and stored at −80°C. Messenger RNA was isolated from frozen tissue samples using a QuantiGene sample processing kit for fresh tissue (Thermo Fisher Scientific). The assay was performed in duplicate using equine specific primers according to the manufacturer's protocol. Samples were analyzed using Luminex Bio-Plex 200 instrumentation (Bio-Rad Laboratories Ltd, Mississauga, Ontario). Results were normalized using expression of hypoxanthine-guanine phosphoribosyltransferase. Data for both horses were averaged and graphed over time. Results are expressed as MFI for the mRNA of each protein.