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Spatial cytokine distribution following traumatic injury

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ABSTRACT

Temporal changes in cytokine concentrations following traumatic injuries have been extensively studied. Less is known regarding spatial differences in cytokine concentrations following traumatic injury. The primary aim of this study was to determine the spatial relationship between cytokines and the zone of injury (ZOI). Muscle and vessel tissues obtained from rats subjected to an open femoral fracture were analyzed to determine if spatial cytokine gradients exist that could potentially be used as biomarkers of the ZOI. Samples were collected at 4 time points following fracture from 3 distinct locations: at the fracture site, 1-cm away from the fracture, and from the opposite leg. The concentrations of IL-6, IL-1α, IL-1ß, IL-2, GM-CSF, TNF-α, and MIP-1α were quantified in each sample. Temporally and spatially regulated variations in cytokine concentrations were found. IL-6 showed the most promise as a ZOI biomarker with statistically different spatial concentrations that were inversely proportional to the distance from the fracture in both tissues. IL-1ß and IL-2 also showed spatial differences in concentration in both tissues, while GM-CSF, MIP-1α, and TNF-α showed spatial differences in vessel samples. These results demonstrate that spatial cytokine gradients exist following traumatic injury, representing potential biomarkers that may be used to define the ZOI.

1. Introduction

The temporal cytokine response to traumatic injury and other physical stresses has been extensively studied in various tissues and experimental models [1–9]. Temporal changes in cytokine concentrations correlate with negative early post-trauma implications such as acute respiratory distress syndrome (ARDS), multiple organ failure (MOF), wound failure, and mortality [5,10–12]. Monitoring cytokine response can aid in assessing the severity of traumatic injuries, as well as provide information to improve the selection of treatment options and their appropriate time course [5–9,13]. Hawskworth et al. demonstrated that cytokine concentrations in wound effluent and tissue gene transcripts are predictive of wound healing outcomes in patients with traumatic combat wound injuries [5], while Pape et al. used cytokines as assessment markers in determining the optimal timing for fracture fixation in patients with blunt injuries [7]. Overall, temporal cytokine responses to traumatic injuries have demonstrated utility as objective predictors of acute wound healing outcomes that can assist surgeons in making appropriate surgical decisions with regard to procedural selection and timing.

While the temporal cytokine response to traumatic injury has been studied in great detail, little is known with regards to the spatial aspect of this response. Previous studies have determined that the local immune environment of traumatic injury sites differ in cytokine concentration in comparison to systemically circulating concentrations [1], but the relationship between tissue cytokine concentrations and physical distance from the injury site remains to be elucidated. Wound healing is a complex, spatially and temporally regulated process of tissue repair resulting in the restoration of the structural and functional integrity of damaged tissue [14,15]. A multitude of cytokines and growth factors present at the wound site regulate all states of the healing process [15]. Cytokines specifically play major roles in the growth, differentiation, migration, angiogenesis, matrix protein deposition, and proliferation facets of wound healing [16–18]. Alterations in cytokine expression patterns are associated with impaired wound healing, suggesting that a correctly coordinated temporal and spatial expression is essential for normal repair [15,19]. Even changes in one cytokine can have a negative effect on wound healing as demonstrated by studies

Abbreviations: ZOI, zone of injury; ARDS, acute respiratory distress syndrome; MOF, multiple organ failure; (IL), Interleukin; GM-CSF, granulocyte macrophage-colony stimulating factor; TNF-α, tumor necrosis factor-α; MIP-1α, macrophage inflammatory protein-1α; PBS, phosphate buffered saline; LLOQ, lower limit of quantitation.

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showing that the knock-out of IL-6 severely impairs wound repair in mice [20].

Since cytokines are proven important regulators of the tissue trauma response, and temporal cytokine expression profiles have shown promise as predictors of healing outcomes, there is a need to determine the spatial relationship between cytokines and the zone of injury (ZOI). The "zone of injury" is recognized as the traumatized area surrounding a wound that is known to extend beyond what is macroscopically observable [21,22]. The physical boundaries of the ZOI are of particular importance to surgeons when performing surgical procedures [22–24]. While the ability to appropriately identify the ZOI can positively influence the treatment of traumatic wounds, there are currently no clinical molecular markers or diagnostic tests capable of defining the ZOI. Here we suggest that cytokines may be exploited as potential biomarkers of the ZOI if they are shown to display spatial relationships to injury sites. Cytokine concentration gradient profiles may be used to spatially and quantitatively assess the state of injured tissue and ultimately may assist in surgical decision making.

In this study, both the spatial and temporal response to blunt trauma was determined. Cytokine concentrations were determined in muscle and vessel samples taken from Sprague–Dawley rats subjected to a femoral fracture. Samples were obtained from 3 distinct locations: directly at the site of fracture, 1-cm away from the fracture, and from the opposite uninjured leg at 0, 6, 24, and 168 h post-fracture. The chosen cytokine targets Interleukin (IL)-6, IL-1α, IL-1ß, IL-2, granulocyte macrophage-colony stimulating factor (GM-CSF), tumor necrosis factor-α (TNF-α), and macrophage inflammatory protein-1α (MIP-1α) are associated with wound healing and cast a wide net on overall activity [5]. From our understanding, this exploratory study is the first to describe both the temporal and spatial tissue cytokine response to traumatic injury.

2. Materials and methods

2.1. Animals

Adult male Sprague–Dawley rats were housed individually with a 12:12 light/dark cycle with ad libitum access to standard rat chow and water. Four time points were studied with 3 replicates each ($N = 3$) for a total of 12 rats for the study. All procedures were performed under the guidelines approved by the West Virginia University Animal Care and Use Committee.

2.2. Femur fracture

After adequate anesthesia, all animals were subjected to a standardized femur fracture on one leg using a custom designed tool in which a weight is dropped in a consistent fashion onto the midshaft of the rat's thigh [25]. This tool delivers a calculated force of 104.80 Newtons, generating a reproducible femur fracture and associated soft tissue injury [25]. Buprenorphine SR was preoperatively administered subcutaneously as an analgesic providing 72 h pain relief. Rats were anesthetized intraperitoneally with Ketamine (80–90 mg/kg) and Xylazine (10–15 mg/kg). This combination of analgesic and anesthetics has previously been identified as the best combination for avoiding significant modulation of cytokine responses in a rat model [26]. Additionally, analgesics and anesthetics were injected into the scruff on the back of the rat's neck, thus there was an equal distance between the injection and both legs. An incision was made to visualize the fracture. A hole was drilled into the proximal femur to allow a 0.045 inch Kirschner wire (K-wire) to be inserted down the intramedullary canal to fix the fracture. The incision was closed starting with the fascia and then using a stainless steel suture on the skin. Rats were subcutaneously administered Yohimbine (2 mg/kg) post-operatively to reverse the Xylazine and were closely observed during recovery for signs of distress.

2.3. Sample preparation

Three rats were sacrificed at each of 4 time points (0, 6, 24, and 168 h post-fracture), and samples were collected immediately following sacrifice. For the 0 h time point, rats were sacrificed immediately following the fracture. Rats were anesthetized intraperitoneally with Ketamine (80–90 mg/kg) and Xylazine (10–15 mg/kg). One cc of Euthasol was then administered via intracardiac puncture. The individual tissue types, vessel and muscle, were identified grossly with the aid of an operating microscope, and collected by a vascular surgeon; these tissues both are intimate to the femur and easily found. The tissues were gently dissected from their fascial attachments and passed off of the operating field. Blood vessel and muscle tissue were harvested from the following 3 locations: at the site of the fracture, 1.0 ± 0.2 cm away from the site of fracture, and from the leg opposite to the fractured leg. Fig. 1 illustrates the sampling locations. Samples were immediately rinsed with ice cold phosphate buffered saline (PBS), snap frozen, and stored at −80 °C. Protein extraction was achieved using methods adapted from those of Hulse et al. [27]. Samples were ground cryogenically and then lyophilized. For analyses, 2–3 mg of lyophilized tissue sample was then thawed for 10 min at 4 °C in 800 µl (muscle samples) or 650 µl (vessel samples) of cell lysis buffer (Bio-Rad, Hercules, CA) containing 20 mM phenylmethylsulfonyl fluoride (Sigma, St. Louis, MO). Thawed samples were then vortexed for 1–3 s and homogenized with 3 rapid pulses using a model 100 ultrasonic dismembrator (Fisher Scientific, Pittsburgh, PA). Samples were vortexed for 1–3 s and centrifuged at 5000*g* for 5 min at 4 °C. The supernatant was collected and total protein concentration was determined using the RCDC protein assay (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Absorbance values were determined using an Infinite M1000 plate reader (Tecan, Raleigh, NC).

2.4. Cytokine measurement

Sample homogenates were diluted to a total protein concentration of 900 µg/ml with sample diluent (Bio-Rad, Hercules, CA), and assayed for cytokines using the Bio-Plex Pro multiplexed magnetic bead-based immunoassay reagent kit along with the Bio-Plex 200 suspension array system and Pro II Wash Station (Bio-Rad, Hercules, CA) according to the manufacturer's instructions.

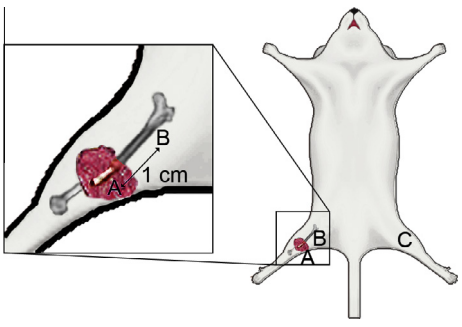

Fig. 1. Sampling locations following femur fracture. Muscle and vessel samples were obtained from three different locations: (A) directly at the fracture site (B) 1-cm away from the fracture site and (C) from the opposite un-injured leg.