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The impact of cytokine responses in the intra- and extracellular signaling network of a traumatic injury

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ABSTRACT

Investigations of cellular responses involved in injury and repair processes have generated valuable information contributing to the advancement of wound healing and treatments. Intra- and extracellular regulators of healing mechanisms, such as cytokines, signaling proteins, and growth factors, have been described to possess significant roles in facilitating optimal recovery. This study explored a collection of 30 spatiotemporal responses comprised of cytokines (IL-1 α , IL-1 β , IL-2, IL-6, TNF- α , MIP-1 α), intracellular proteins (Akt, c-Jun, CREB, ERK1/2, JNK, MEK1, p38, p53, p90RSK), phosphorylated proteins (p-Akt, p-c-Jun, p-CREB, p-ERK1/2, p-GSK-3 α / β , p-HSP27, p-I κ B α , p-JNK, p-MEK1, p-p38, p-p70S6K, p-p90RSK, p-STAT2, p-STAT3), and a protease (Caspase-3), measured in skeletal muscle tissue following a traumatic injury (rodent Gustilo IIIB fracture). To optimize the analysis of context-specific data sets, a network centrality parameter approach was used to assess the impact of each response in relation to all other measured responses. This approach identified proteins that were substantially amplified and potentially central in the wound healing network by evaluation of their corresponding centrality parameter, radiality. Network analysis allowed us to distinguish the progression of healing that occurred at certain time points and regions of injury. Notably, new tissue formation was proposed to occur by 168 h post-injury in severely injured tissue, while tissue 1-cm away from the site of injury that experienced relatively minor injury appeared to exhibit signs of new tissue formation as early as 24 h post-injury. In particular, hallmarks of inflammation, cytokines IL-1 β , IL-6, and IL-2, appear to have a pronounced impact at earlier time points (0–24 h post-injury), while intracellular proteins involved in cell proliferation, differentiation, or proteolysis (c-Jun, CREB, JNK, p38, p-c-Jun; p-MEK1, p-p38, p-STAT3) are more significant at later times (24–168 h). Overall, this study demonstrates the feasibility of a network analysis approach to extract significant information and also offers a spatiotemporal visualization of the intra- and extracellular signaling responses that regulate healing mechanisms.

1. Introduction

The elucidation of molecular and signaling responses involved in traumatic injuries has gained considerable interest for potential treatments that may enhance the healing process. Previous studies have investigated the roles of cytokines, chemokines, growth factors [1], or signaling proteins [2] involved in the characteristic stages of wound repair: hemostasis, inflammation, tissue formation, and remodeling [3]. However, certain factors can influence the measured responses, such as

varying classes and severities of injuries (e.g. fractures, minor cuts) and different tissue types (e.g. cutaneous tissue, bone, or myofibroblasts), which all appear to have distinct repair mechanisms [4]. Additionally, the measured response may be affected by temporal factors (e.g. time post-injury), as well as the spatial distribution of the zones of injury [5]. An increasing collection of intra- and extracellular regulators continues to emerge as prospective contributors of healing, allowing researchers to examine signaling pathways [6,7] or networks [8] involved in repair mechanisms. However, as the number of injury biomarker grows, it can

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become difficult to interpret large datasets and translate the results into information useful to practitioners and researchers.

Complex datasets, particularly those describing biological responses, may benefit from a data-driven mathematical modelling or a network analysis approach [9]. Leading bioinformatic software programs specializing in network analysis include Ingenuity Pathway Analysis (IPA), Cytoscape, and Pathway Studio, to name a few. These software programs allow for the construction of molecular networks and signaling pathways, or the identification of involved molecular functions and even disease states using experimental data [10]. Network analyses of biochemical responses are best recognized as tools to evaluate protein-protein interactions, construct signal transduction pathways, or elucidate metabolic patterns in order to better understand how entire systems respond when perturbed by detrimental events [11]. In particular, taking a graph theory approach to evaluate mathematical relationships among the components in a network can aid in sorting through and dissecting large networked datasets. Network analytics, such as centrality parameters, assigns a value to each node (measured response) that may describe how central one node is, relative to all other nodes in the system. Examples of centrality parameters include degree (number of nodes directly connected to a given node), diameter (the distance between the two furthest nodes in the network), or radiality (sum of shortest paths between a given node and all other nodes, normalized to the network diameter) [12]. Node centrality has been used to examine biological networks, such as protein-protein interactions resulting from chronic diseases [13] or phosphorylation responses elicited by toxic exposures [14]. In these studies, centrality parameters were used to identify biological components that were highly impactful, relative to all other measured responses in the network. A node with a high centrality can be interpreted as having a greater degree of likeness to all other nodes, while a node with a low centrality can be construed as having a more peripheral position in the network (behaving differently from all other nodes in the group) [12].

In this study, 30 intra- and extracellular responses were measured in skeletal muscle tissue following a traumatic injury, in an effort to expand on our understanding of the spatial and temporal signaling changes that occur during wound healing. Cytokine, total intracellular protein, phosphorylated intracellular protein, and Caspase-3 levels were observed at varying time points (0, 6, 24, 168 h post-injury) and locations of injury (at the site of injury, 1-cm away). This complete dataset was examined with IPA to provide a comparison between a recognized software application, often used for analyzing and integrating large biological datasets, and the proposed method. Eight different networks of all responses were analyzed for each spatio-temporal condition (e.g. At the site of injury, 0 h) in order to obtain the centrality parameter, radiality, for each node. This approach was used to assess the value of each node, relative to all other nodes, under various temporal and spatial conditions. Radiality values were then used to identify nodes that had particularly distinctive responses, potentially holding a prominent role in driving the mechanisms of wound healing. This method of analysis allows for an unbiased comparison of all observations regardless of biological hierarchical assignments (e.g. extracellular cytokines and intracellular phosphorylated proteins).

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. Three sampling (injury) locations were studied at four time points, with 3 replicates each ($N = 3$) for a total of 12 rats for the study. All procedures approved by the West Virginia University Animal Care and Use Committee.

Table 1

Intra- and Extracellular biomolecules measured after traumatic injury.

	Target
Total intracellular protein	Akt c-Jun CREB ERK1/2 JNK MEK1 p38 p53 p90RSK
Phosphorylated protein (site)	p-Akt (Ser473) p-c-Jun (Ser63) p-CREB (Ser133) p-ERK1/2 (Thr202/Tyr204, Thr185/Tyr187) p-GSK-3 α / β (Ser21/Ser9) p-HSP27 (Ser78) p-I κ B α (Ser536) p-JNK (Thr183/Tyr185) p-MEK1 (Ser217/Ser221) p-p38 (Thr180/Tyr182) p-p70S6K (Thr421/Ser424) p-p90RSK (Ser380) p-STAT2 (Tyr689) p-STAT3 (Tyr705)
Cytokine	IL-1 α IL-1 β IL-2 IL-6 TNF- α MIP-1 α
Protease	Caspase-3

2.2. Sample analysis

Intra- and extracellular biomolecule responses were examined after animals were subjected to a standardized femur fracture (Gustilo IIIB), as described in Currie et al. [5], and Han et al. [15]. Briefly, muscle tissue samples were collected at the respective time points (0 h, 6 h, 24 h, and 168 h post-injury) and injury locations (at the site of the injury, 1-cm away, and in the uninjured leg), rinsed with ice cold phosphate buffered saline, snap frozen, and stored at -80°C . Homogenized tissues were analyzed for 30 targets, including nine intracellular proteins, 14 phosphoproteins, six cytokines, and one protease (listed in Table 1). These targets were chosen based on previously reported molecular responses of injuries in literature, as well as known responses involved cellular processes vital to the healing mechanism (e.g. cell death, cell proliferation, inflammation). The relative abundance of total intracellular protein and phosphoprotein were measured with a Bio-plex kit containing polystyrene, non-magnetic antibody coated beads. Cytokine responses were measured with a Bio-Plex Pro multiplexed magnetic bead-based immunoassay reagent kit and Caspase-3 activity was measured with a fluorescence assay kit from Cayman Chemical (Item No. 10009135). All beads were analyzed using the Bio-Plex 200 suspension array system, along with the Pro II Wash Station (Bio-Rad, Hercules, CA). Fluorescence readings were taken with the Infinite M1000 microplate reader (Tecan US, Raleigh NC, USA).

2.3. Data processing and statistical analysis

Data were analyzed using Prism 5 (GraphPad, San Diego, CA), and SAS JMP Pro 12.0.1 (Carey, NC). Total intracellular protein, phosphoprotein, cytokine, and Caspase-3 abundances measured in muscle tissue at the site of fracture and 1-cm away from the site were normalized to levels found in tissue collected from the uninjured leg. Normalizing to the uninjured leg served as a systemic control, accounting for any responses that were not directly involved in the response to the local

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