Biochemistry and Biophysics Reports 7 (2016) 56–62

Contents lists available at ScienceDirect

Biochemistry and Biophysics Reports

journal homepage: www.elsevier.com/locate/bbrep

Actin exposure upon tissue injury is a targetable wound site-specific protein marker

Erik D. Pendleton [a,1], Challise J. Sullivan [a,1], Henri H. Sasmor [a], Kristy D. Bruse [b], Tifanie B. Mayfield [a], David L. Valente [a], Rachel E. Abrams [a], Richard H. Griffey [a], John Dresios [a,*]

[a] Leidos, Inc., 10260 Campus Point Drive, San Diego, CA 92121, United States
[b] Lovelace Respiratory Research Institute, 2425 Ridgecrest Drive SE, Albuquerque, NM 87108, United States

ARTICLE INFO

Article history:
Received 9 November 2015
Received in revised form
5 April 2016
Accepted 17 May 2016
Available online 18 May 2016

Keywords:
Injury
Wound
Bleeding
Hemorrhage
Protein marker
Actin

ABSTRACT

Background: Identification of wound-specific markers would represent an important step toward damaged tissue detection and targeted delivery of biologically important materials to injured sites. Such delivery could minimize the amount of therapeutic materials that must be administered and limit potential collateral damage on nearby normal tissues. Yet, biological markers that are specific for injured tissue sites remain elusive.
Methods: In this study, we have developed an immunohistological approach for identification of protein epitopes specifically exposed in wounded tissue sites.
Results: Using *ex-vivo* tissue samples in combination with fluorescently-labeled antibodies we show that actin, an intracellular cytoskeletal protein, is specifically exposed upon injury. The targetability of actin in injured sites has been demonstrated *in vivo* through the specific delivery of anti-actin conjugated particles to the wounded tissue in a lethal rat model of grade IV liver injury.
Conclusions: These results illustrate that identification of injury-specific protein markers and their targetability for specific delivery is feasible.
General significance: Identification of wound-specific targets has important medical applications as it could enable specific delivery of various products, such as expression vectors, therapeutic drugs, hemostatic materials, tissue healing, or scar prevention agents, to internal sites of penetrating or surgical wounds regardless of origin, geometry or location.

1. Introduction

There are a milieu of biochemical and physiological events at a wound site due to structural changes resulting from cell lysis or tissue rupture and the associated displacement of intracellular or extracellular proteins from their natural locations. Studies in model organisms, such as zebrafish, demonstrated that myocellular injury leads to expression of specific markers, such as Xin-actin-binding repeat-containing proteins (Xirp), with a potential role in cytoskeletal reorganization and muscle regeneration [13]. Other studies have shown that injury of the blood vessel wall and surrounding extracellular matrix by mechanical rupture or heat results in irreversible unfolding of collagen from its native state [22], generating signals for induction of arteriolar vasodilation and increase in blood flow to the injured area [10]. In addition, necrotic cells resulting from tissue injury can be detected by the immune system through recognition of damage-associated molecular patterns (DAMPs) by dendritic cell receptors, leading to inflammatory response and tissue repair [2,16]. Identification of targetable wound-specific markers would represent a viable mechanism for the detection and specific delivery of biologically important materials to wound surfaces, such as therapeutic or hemostatic payloads. Additionally, identification of such targets might be advantageous in minimizing administered dosages, improve the availability due to increased local delivery, and avoid potential deleterious interactions with nearby healthy tissue. Wound targeting would also enable binding to non-compressible, asymmetrically exposed internal organ areas as a result of penetrating or surgical wounds, irrespective of geometry and location. To date, however, biological epitopes that are specific to injured tissue sites remain largely unknown.

The objective of this study was to identify protein markers that become rapidly exposed in wounded animal tissues and might be

* Correspondence to: Leidos, Inc., 10260 Campus Point Drive, SanDiego, CA 92121. U.S.A.
E-mail address: john.dresios@leidos.com (J. Dresios).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.bbrep.2016.05.013

useful for injury site detection and/or targeted delivery of therapeutic products to the trauma surface, irrespective of wound shape and/or location. We hypothesized that rapid site detection and wound-specific delivery of medical products would require recognition of structural, physical and/or biochemical signatures unique to the damaged tissue that are present at the wound site immediately upon injury. To this end, we developed a method for screening and identifying proteins that are rapidly and specifically exposed in injured animal tissues. Using this approach, we identified an abundant cytoskeletal protein as a wound marker and evaluated its targetability for localized delivery of materials at the site of the wound both *ex vivo* and *in vivo*.

2. Materials and methods

2.1. Reagents

2-(N-morpholino)ethanesulfonic acid (MES) buffer, Bovine serum albumin (BSA) and NaN_3 were purchased from Sigma-Aldrich Corporation, MI. Fetal Bovine Serum (FBS) Hyclone, 10% Neutral Buffered Formalin, Tissue Tek optimum cutting temperature (O. C. T.) compound, sucrose, and Fluorescent Mounting Media were purchased from VWR International, PA. Tween-20 10% was purchased from Bio-Rad Laboratories, CA. Carboxylated fluorescent (505/515) 2 μm beads were purchased from Invitrogen, CA. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysulfosuccinimide (Sulfo-NHS) were purchased from Thermo Scientific, IL. DAPI (4′,6-Diamidino-2-Phenylindole, Dihydrochloride) was purchased from Thermo Fisher Scientific, MA.

2.2. Antibodies

Anti-β-Actin-Fluorescein isothiocyanate (FITC) mouse monoclonal antibody and mouse IgG1 anti-β-actin were purchased from Sigma-Aldrich Corporation, MI. Alexa-488 donkey anti-mouse IgG and goat anti-mouse IgG1 were purchased from Jackson ImmunoResearch, PA. Anti-collagen I, anti-Elastin, anti-Entactin and mouse IgG1 anti-human IgM were purchased from AbCam, MA. Alexa 488 anti-S6 ribosomal 54D2 antibody was purchased from Cell Signaling Technology, MA.

2.3. Antibody fluorescent labeling

Anti-Collagen I, anti-Elastin, anti-Entactin and mouse IgG1 anti-human IgM were FITC-labeled using N-Hydroxysuccinimide (NHS)-Fluorescein (Thermo Fisher Scientific, MA) according to manufacturer's recommendations with slight modifications. Briefly, the antibody sample (1 mg/mL) was mixed with freshly reconstituted NHS-Fluorescein in DMSO and the labeling reaction was incubated overnight (12–16 h) at 4 °C. The labeled antibody was then separated from excess fluorescein reagent utilizing MicroSpin G-25 columns (GE Healthcare Bio-Sciences Corporation, PA) per the manufacturer's recommended conditions. Protein concentration in the eluant was estimated utilizing the Pierce™ BCA Protein assay kit (Thermo Fisher Scientific, MA). The efficiency of antibody fluorescence labeling was measured using a Mithras LB 940 multimode microplate reader (Berthold Technologies, TN). Antibody preparations used in these studies had similar specific fluorescence intensities.

2.4. Ex vivo tissue samples

Rat tissues for *ex vivo* experiments were harvested from healthy male Sprague-Dawley rats and immediately snap frozen in liquid nitrogen. Rat liver tissue was purchased from BioChemed Services (Winchester, VA) or Analytical Biological Services, Inc. (Wilmington, DE). Rat kidney and rat spleen tissues were purchased from Innovative Research, Inc. (Novi, MI). Rat skeletal muscle tissue was purchased from BioreclamationIVT (Liverpool, NY). Bovine whole blood with sodium heparin anticoagulant was purchased from Innovative Research, Inc. (Novi, MI).

2.5. Tissue injury ex vivo and wound targets screening

The rat tissue samples (approximately $2 \times 2 \times 2\ cm^3$ in size) were thawed at 4 °C and, depending on the type of tissue, dissection scissors, forceps, or razor blades were used to create an X-shaped wound. The wounds covered the majority of the surface area, measuring approximately 1.5 cm in diameter and cutting all the way through the tissue, top to bottom. Immediately following injury, the tissue samples were placed in 5 mL cold blocking solution (10% FBS in PBS) and incubated for 1 h at 4 °C. FITC-labeled antibodies against the potential injury-specific target epitopes (or anti-mouse IgG as negative control) were diluted in blocking solution (500 μL at a final concentration of 0.05 μg/μL) and added to tissue samples followed by incubation at 4 °C for 1 h. After antibody incubation, 1 mL cold PBST (0.1% Tween-20 in PBS) was added to the wound to remove unbound antibody (total of two washes). The samples were fixed by placement in a 35 mL cold 10% Neutral Buffered Formalin (VWR International, PA) followed by overnight fixation at 4 °C. Samples were then placed in 35 mL cold 30% Sucrose/PBS overnight at 4 °C for cryoprotection. The samples were mounted into cryomolds in O.C.T. medium, with the X-shaped wound facing the bottom of the cryomold, and placed in a −70 °C freezer. Frozen blocks were shipped to Molecular Diagnostic Services (CA) for cryosectioning using a microtome to 10-micron thick sections, and mounting of the tissue sections onto microscope slides. For analysis, slides were warmed to room temperature and submerged in PBS for 5 min before applying Fluorescent Mounting Media (VWR International, PA) and coverslips. Tissue slides were viewed using an Olympus IX51 inverted microscope (Olympus, MA). Images were captured using a MicroFire™ digital microscope imaging camera and Picture Frame™ software (Optronics Engineering Ltd., TX). For nuclear staining, tissue sections were incubated with DAPI [(100 ng/mL in phosphate-buffered saline (PBS))] in dark for 5 min at room temperature prior to fluorescence imaging. For testing antibody binding in the presence of blood, wounded tissue samples were immersed in bovine blood and incubated for 1 h with FITC-labeled antibodies (anti-β-actin or IgG) previously diluted in heparinized bovine blood (antibody final concentration 0.05 μg/μL). The rest of the experimental processes were performed essentially as described above.

For quantitative analysis of the specific signal increase within the injury sites relative to uninjured tissue, images from tissues treated with fluorescently labeled antibodies were converted to grayscale and multiple representative rectangular regions were selected from the wounded surface as well as the neighboring healthy tissue (Supplemental Fig. 1). Quantitative analysis was performed as an aggregate for all selected wounded regions. Same analysis was performed for the selected healthy tissue regions to represent the background. The resulting mean values and standard deviations were calculated and graphically represented using Matlab R2013b software (MathWorks, Natick, MA).

2.6. Microbead-antibody conjugation

Yellow-green fluorescent carboxylate-modified microspheres (nominal bead diameter of two microns) (Thermo Fisher Scientific, MA) were washed by mixing 500 μL of beads suspension (2%) with