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Citrullinated histone 3 causes endothelial barrier dysfunction

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ABSTRACT

Circulating components of neutrophil extracellular traps (NETs), especially histones, are associated with tissue injury during inflammatory conditions like sepsis. Commonly used as a NET biomarker, citrullinated histone 3 (H3Cit) may also functionally contribute to the NET-associated inflammatory response. To this end, we sought to examine the role of H3Cit in mediating microvascular endothelial barrier dysfunction. Here we show that H3Cit can directly contribute to inflammatory injury by disrupting the microvascular endothelial barrier. We found that endothelial responses to H3Cit are characterized by cell-cell adherens junction opening and cytoskeleton reorganization with increased F-actin stress fibers. Several signaling pathways often implicated in the transduction of hyperpermeability, such as Rho and MLCK, did not appear to play a major role; however, the adenylyl cyclase activator forskolin blocked the endothelial barrier effect of H3Cit. Taken together, the data suggest that H3Cit-induced endothelial barrier dysfunction may hold promise to treat inflammatory injury.

1. Introduction

Circulating extracellular histones are associated with tissue injury during the systemic inflammatory response to infection [1] or trauma [2]. While histones are generally located within the cell nucleus, they can be released into the circulation or tissues upon cell injury or programmed death, especially during the release of neutrophil extracellular traps (NETs) [3]. NETosis is a unique cell death mechanism initiated by the peptidylarginine deiminase 4 (PAD4)-mediated citrullination of histone 3, which causes decondensation of chromatin and subsequent release of a DNA-web containing histones, proteases, and other granular or cytoplasmic contents [3,4]. Components of NETs, including histones, have been shown to increase in the circulation during many inflammatory conditions including sepsis [5], acute lung injury [6,7], autoimmune diseases [8], and cancer [9], damaging vasculature by promoting coagulopathy [10,11] and vascular barrier dysfunction [12,13].

Representing a common endpoint in a number of inflammatory injuries, endothelial barrier dysfunction causes fluid leakage and leukocyte infiltration that leads to tissue damage and multiple organ failure [14,15]. Elucidating the molecular mechanisms of endothelial barrier regulation is needed to further develop targeted therapies in inflammatory disease. While there is no specific marker of NETs, citrullinated histone 3 (H3Cit) has been recognized as a key component to determine the presence of NETs and has been proposed as an inflammatory biomarker [16], as it has been shown to increase in mice and humans during various inflammatory states [17,18]. Interestingly, H3Cit could have causative effects on tissue injury, as it has been shown that injection of either pharmacological PAD inhibitors [18,19] or H3Cit antibody [20] can improve outcomes of systemic inflammation. To this end, we sought to determine the direct effects H3Cit might have on the endothelial barrier.

2. Materials and methods

2.1. Animals

Mice used in these studies were male C57BL/6J purchased from Jackson Laboratory. Genotyping was performed by Transnetyx using real-time PCR. Mice were maintained under a 12/12-h light/dark cycle with food and water *ad libitum*. All animal studies were approved by the University of South Florida Institutional Animal

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Care and Use Committee and was performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

2.2. Intravital microscopic analysis of protein transvascular flux

To examine plasma protein flux across mesenteric microvessels [21], mice (10–20g) were anesthetized with an intramuscular injection of urethane (1.75 g/kg) and shaved at the abdomen. The jugular vein was cannulated for IV infusion of solutions. A midline laparotomy was performed, and the mesentery was exteriorized over an optical stage for microscopic observation. The microcirculation of the mesentery was imaged using a Nikon Eclipse E600FN microscope with Evolve 512 digital camera (Photometrics, AZ, USA). Mice were given an IV bolus of fluorescein isothiocyanate conjugated bovine albumin (FITC-albumin) at 100 mg/kg followed by continuous infusion of 0.15 mg/kg/min to maintain a constant plasma concentration. Postcapillary venules were selected for analysis of FITC-albumin flux and stimulated with 10 μg/mL recombinant H3Cit or vehicle control. FITC-albumin leaking into extra-vascular space was accumulated over time. Fluorescent images were acquired every 5 min for 1 h. Protein flux was quantified using the formula $IOI_{Rel}=(I_i-I_o)/I_i$, where I_i = intensity inside the vessel and I_o = intensity outside the vessel.

2.3. HUVEC cell culture

Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza and grown in Endothelial Cell Basal Medium supplemented with EGM-2 MV Bulletkits (Lonza, MD, USA), or from PromoCell and grown in Endothelial Cell Growth Medium 2 supplemented with SupplementMix C-39216 (PromoCell GmbH, Heidelberg, Germany). Cells were seeded onto 0.1% gelatin-coated plates and incubated in 5% CO_2 humidified incubator at 37 °C for 2–3 days past confluence for use in experimental assays.

2.4. Transendothelial electrical resistance

HUVECs were seeded onto 8W10E + PET electrode arrays to be used with an Electric Cell-Substrate Impedance Sensing (ECIS) system (Applied Biophysics, Troy, NY). Transendothelial electrical resistance (TER) was continuously recorded over time as an indicator of cell-cell adhesive barrier function [22]. Cells were stimulated with human recombinant H3Cit (Item No. 17926; Cayman Chemical, Ann Arbor, MI) or vehicle control (0.1% BSA in PBS) with or without inhibitors. Representative tracings are presented normalized to baseline. Change in resistance was quantified by subtracting the lowest resistance value from the baseline resistance value of each ECIS well and averaging maximum change from baseline at each concentration. Inhibitors used in this study include Rho kinase inhibitor Y27632 (Cayman), Rho inhibitor Rhosin (Calbiochem), and MLCK inhibitor peptide 18 (Cayman). Barrier enhancing agent forskolin (MP Biomedicals) was used as a pharmacological therapeutic.

2.5. Cell viability assay

LIVE/DEAD™ Viability/Cytotoxicity Kit, for mammalian cells (Cat. No L3224; Invitrogen) was used per manufacturer's instructions. Absorbance was read at 530 nm (calcein AM) to determine live cells and at 645 nm (ethidium homodimer-1) to determine dead cells.

2.6. Immunofluorescence confocal microscopy

HUVECs were seeded onto coverslips and treated with human recombinant H3Cit or vehicle control for 1 min for immunofluorescence staining as we've previously described. For adherens junction staining, cells were fixed with 4% paraformaldehyde for 10 min at room temperature, washed twice with PBS, blocked with 10% donkey serum in PBS for 1 h, and labeled with VE-cadherin (D87F2) XP® Rabbit mAb #2500 (CST; 1:500) overnight at 4 °C in a humidified chamber. The next day, cells were washed with PBS, incubated with donkey anti-rabbit IgG Alexa Fluor 488 (Invitrogen; Cat #R37118) for 1 h at room temperature, washed again with PBS, and mounted to slides with ProLong Diamond Antifade Mountant with DAPI (Life Technologies). Fluorescent confocal images were captured with an Olympus FV1200 Laser Scanning Confocal Microscope. VE-cadherin intensity was analyzed with FIJI (version 2.0.0-rc-65/1.51w) software by taking the sum of Z-stack (6 slices), subtracting the background (radius = 10), applying Gaussian blur (1.00), converting to mask, and analyzing the particles (size 50-Infinity, circularity 0–0.25) per field of view (~212 μm^2). Actin staining was performed in the same manner, except cells were permeabilized with 0.1% PBS-T for 5 min before blocking and were stained with Alexa Fluor 568 Phalloidin (F-actin) and Alexa Fluor 488 DNAse I (G-actin) for 20 min at room temperature. Intensity of each type of actin was analyzed using FIJI (version 2.0.0-rc-65/1.51w) software by taking the sum of Z-stack (10 slices) and measuring total intensity per field of view (~318 μm^2), represented as F:G-actin ratio.

2.7. Statistical analysis

Statistics were performed using GraphPad Prism (version 6.0f/7.0d). Pairwise-comparisons were made using unpaired two-tailed *t*-test and group-wise comparisons were made using ordinary one-way ANOVA with Tukey's post hoc multiple comparisons test. Statistical significance was defined as $p \leq 0.05$.

3. Results and discussion

3.1. H3Cit causes microvascular leakage and endothelial barrier dysfunction without cell death

To determine the direct effects of H3Cit in the microvascular endothelial barrier, we used intravital microscopy to examine the protein transvascular flux and found that H3Cit (10 μg/mL) caused extravasation of fluorescent-labeled albumin across microvessels (Fig. 1). Furthermore, we used the ECIS system to measure TER, an indicator of cell-cell adhesive barrier strength and found that stimulation of HUVECs with H3Cit decreased TER in a concentration-dependent manner, indicating endothelial barrier dysfunction (Fig. 2A and B); this response was not due to cell death (Fig. 2C). Taken together, we concluded that H3Cit is capable of disrupting the microvascular endothelial barrier without causing cell toxicity.

3.2. H3Cit-mediated endothelial barrier dysfunction is not dependent on Rho or MLCK signaling pathways

Limited information is available regarding the receptor and signaling mechanisms underlying histone-induced endothelial injury. Similar to other components of NETs, such as extracellular DNA, histones can be considered to act as a pattern recognition molecule. So far, cellular pathways seemingly activated by extracellular histones include toll-like receptor [23,24] and inflammasome [25,26] signaling, as well as membrane integration and calcium influx [2,27]. However, modifications of histones can affect their size, charge, and structure [28], which could change the way they interact with the endothelium. For example, we were unable

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