Journal of Immunological Methods xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Journal of Immunological Methods

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

Research paper

Colocalization of neutrophils, extracellular DNA and coagulation factors during NETosis: Development and utility of an immunofluorescence-based microscopy platform

Laura D. Healy [a,*], Cristina Puy [b], Asako Itakura [a], Tiffany Chu [b], David K. Robinson [b], Alan Bylund [b], Kevin G. Phillips [b], Elizabeth E. Gardiner [c], Owen J.T. McCarty [a,b]

[a] Department of Cell, Developmental & Cancer Biology, Oregon Health & Science University, Portland, OR, USA
[b] Department of Biomedical Engineering, Oregon Health & Science University, Portland, OR, USA
[c] Department of Cancer Biology and Therapeutics, John Curtin School of Medical Research, Australian National University, Canberra, Australia

ARTICLE INFO

Article history:
Received 1 March 2016
Received in revised form 12 May 2016
Accepted 6 June 2016
Available online xxxx

Keywords:
Neutrophil extracellular traps
Coagulation factors
Fluorescence microscopy
Biophysical measurement

ABSTRACT

Background: Neutrophils, the most populous innate immune cell type, are the first responders to sites of infection and inflammation. Neutrophils can release their DNA to form extracellular traps (NETs), webs of DNA and granular proteases that contribute to pathogen clearance and promote thrombus formation. At present, the study of NETs is in part limited to the qualitative analysis of fluorescence microscopy-based images, thus quantification of the interactions between NETs and coagulation factors remains ill-defined.
Aim: Develop a quantitative method to measure the spatial distribution of DNA and colocalization of coagulation factor binding to neutrophils and NETs utilizing fluorescence-based microscopy.
Approach: Human neutrophils were purified from peripheral blood, bound to fibronectin and treated with the PKC-activator phorbol myristate acetate (PMA) to induce neutrophil activation and NETs formation. Samples were incubated with purified coagulation factors or plasma before staining with a DNA-binding dye and coagulation factor-specific antibodies. The spatial distribution of DNA and coagulation factors was imaged via fluorescence microscopy and quantified via a custom-built MATLAB-based image analysis algorithm. The algorithm first established global thresholding parameters on a training set of fluorescence image data and then systematically quantified intensity profiles across treatment conditions. Quantitative comparison of treatment conditions was enabled through the normalization of fluorescent intensities using the number of cells per image to determine the percent and area of DNA and coagulation factor binding per cell.
Results: Upon stimulation with PMA, NETs formation resulted in an increase in the area of DNA per cell. The coagulation factor fibrinogen bound to both the neutrophil cell body as well as NETs, while prothrombin, FX and FVIIa binding was restricted to the neutrophil cell body. The Gla domain of FX was required to mediate FX-neutrophil binding. Activated protein C (APC), but not Gla-less APC, bound to neutrophil cell bodies and NETs in a punctate manner. Neither FXIIa nor FXIa were found to bind either neutrophil cell bodies or NETs. Fibrinogen binding was dependent on extracellular DNA, while FX and APC required phosphatidylserine exposure for binding to activated neutrophils.
Conclusions: We have developed a quantitative measurement platform to define the spatial localization of fluorescently-labeled coagulation factor binding to neutrophils and extracellular DNA during NETosis.

1. Introduction

Polymorphonuclear leukocytes, or neutrophils, are the most abundant circulating white blood cell type in humans, and play an indispensable role in innate immune host defense. Conversely, in the setting of inflammatory disease, excessive neutrophil activation has been shown to contribute to thrombotic complications. At sites of inflammation, activated neutrophils release intracellular granule proteins and chromatin that together form neutrophil extracellular traps (NETs) in an alternative form of cell death (Brinkmann et al., 2004; Fuchs et al., 2007).

Abbreviations: NETs, neutrophil extracellular traps; FXII, factor XII; FXI, factor XI; FX, factor X; FX-GD, Gla-less FX; FVIIa, factor VIIa; APC, Activated protein C; APC-GD, Gla-less APC; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; Gla-domain, γ-carboxyglutamic acid domain; HK, high molecular weight kininogen; AnxV, Annexin V.

* Corresponding author at: Department of Cell, Developmental & Cancer Biology, 3303 SW Bond Ave, Center for Health & Healing 13B, Oregon Health & Science University, Portland, OR 97239, USA.
E-mail address: healyl@ohsu.edu (L.D. Healy).

http://dx.doi.org/10.1016/j.jim.2016.06.002
0022-1759/

These decondensed chromatin fibers are decorated with antimicrobial proteins and proteases including elastase, cathepsin G and myeloperoxidase (MPO), forming a physical trap to sequester and clear pathogens, considered an additional host defense mechanism (McDonald et al., 2012; Mócsai, 2013). In addition to these microbial clearance functions, NETs have been shown to promote thrombin generation, fibrin formation and platelet activation in both in vitro and in vivo models to promote thrombus formation (Gould et al., 2014; Massberg et al., 2010; Semeraro et al., 2011). Previous studies have indicated that fibrinogen and coagulation factors of the intrinsic pathway associate with the DNA, histones or proteases comprising NETs (Fuchs et al., 2010; Oehmcke et al., 2009; von Brühl et al., 2012). However, it is unclear whether NETs are involved in an active or passive manner in promoting thrombus formation. Specifically, it is unclear whether NETs directly promote activation of coagulation factors, such as factor XII, or act as a scaffold for the assembly and activation of coagulation factors. In contrast, it is possible that NETs serve to bind and sequester active serine proteases, analogous to the binding and inactivation of thrombin on fibrin. These questions are difficult to address experimentally as they require systems that permit quantitative assessment of the binding, assembly and activation of coagulation factors on NETs. Here, we report on the development of a custom-built MATLAB-based image analysis algorithm that coupled with fluorescence microscopy-based images can provide quantitative analysis and spatial localization of coagulation factors binding to neutrophils as they undergo NETosis.

2. Materials and methods

2.1. Reagents

Activated factor XII(a), factor XIa, factor X, FX-GD, factor VIIa, protein C, APC-GD, prothrombin, fibrinogen, and anti-human mouse antibodies to protein C/APC (AHPC-5071), FVII (AHFVII-5031), FX (AHX-5050), and prothrombin (AHP-5013) were purchased from Hematologic Technologies Inc. (Essex Junction, VT, USA). Activated protein C (APC) was a gift from Dr. András Gruber (Oregon Health & Science University, Portland, OR, USA). Polymorphprep was from Axis-Shield PoC AS (Oslo, Norway). Rabbit polyclonal antibody to fibrinogen was from MP Biomedicals (Santa Ana, CA). Mouse monoclonal antibody to factor XII heavy chain (sc-59517) was from Santa Cruz Biotech (Dallas, TX). The cell-permeable DNA dye Hoechst 33342 was from Invitrogen (Grand Island, NY). Alexa Fluor conjugated anti-mouse antibodies and rabbit polyclonal anti-histone H3 (ab5103) were from Abcam (Cambridge, MA). The antibody 1A6, against the A3 domain of human FXI was generated as described (Tucker et al., 2009). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Preparation of human neutrophils

Human neutrophils were purified as previously described (Itakura and McCarty, 2013). Briefly, human blood was drawn in accordance with an Oregon Health & Science University Institutional Review Board-approved protocol from healthy donors by venipuncture into citrate-phosphate-dextrose (1:7 vol/vol). Blood was layered over an equal volume of Polymorphprep and centrifuged at 500*g* for 45 min at 18 °C. The lower layer containing neutrophils was subsequently collected and washed with HBSS by centrifugation at 400*g* for 10 min. To remove red blood cells from the sample, the pellet was resuspended in sterile H_2O for 30 s, followed by immediate addition of 10× PIPES buffer (250 mM PIPES, 1.1 mM $CaCl_2$, and 50 mM KCl, pH 7.4). After centrifugation at 400*g* for 10 min, the pellet was resuspended in buffer (HBSS containing 2 mM $CaCl_2$, 2 mM $MgCl_2$ and 1% wt/vol BSA).

2.3. Immunofluorescence microscopy

Purified human neutrophils (2×10^6/mL) were stimulated with HBSS or PMA (10 nM) for 3 h at 37 °C on fibronectin-coated glass coverslips. For initial colocalization experiments, cell samples were washed and treated with vehicle (HBSS buffer), fibrinogen (2.6 mg/mL), prothrombin (FII, 100 μg/mL), FX (10 μg/mL), FX-GD (10 μg/mL), protein C (300 nM), APC (300 nM), APC-GD (300 nM), FVIIa (300 nM), FXIa (20 μg/mL), FXIIa (20 μg/mL) in the presence of HK (20 μg/mL) and $ZnCl_2$ (25 μM) was then incubated for 15 min with the cell samples at 37 °C. For select experiments, cell samples were washed and treated with vehicle (HBSS buffer), DNase I (10,000 U/mL), RGDS (20 μM), Annexin V (10 μg/mL) for 10 min at 37 °C. In these experiments, cell samples were then washed and treated with vehicle (HBSS buffer containing BSA), fibrinogen (2.6 mg/mL), FX (10 μg/mL), and APC (300 nM) for 15 min with the cell samples at 37 °C. In select experiments cells were otherwise incubated with platelet-poor plasma or vehicle (HEPES containing 2 mM $CaCl_2$, 2 mM $MgCl_2$ and 0.1% BSA) (1:1) for 15 min at 37 °C.

Subsequently, samples were washed with PBS and fixed with 4% PFA followed by incubation with blocking buffer (PBS containing 10% FBS and 5 mg/mL Fraction V BSA). Cells and coagulation factors were stained with anti-FXII (50 μg/mL), 1 A6 (anti-FXI, 1:50), anti-PC/APC (100 μg/mL), anti-FVII (100 μg/mL), anti-fibrinogen (1:100), anti-FX (50 μg/mL) or anti-prothrombin (50 μg/mL) in blocking buffer at 4 °C overnight. Secondary goat anti-rabbit IgG antibody conjugated with AlexaFluor 488 (1:500) and goat anti-mouse IgG antibody conjugated with AlexaFluor 546 (1:500) and Hoescht 33342 (10 μg/mL) in blocking buffer were added and incubated for 2 h in the dark. Coverslips were mounted onto glass slides and visualized with a Zeiss Axiovert fluorescence microscope.

2.4. Preparation of endothelial cells

Human umbilical endothelial cells (HUVECs) were grown to confluency on glass coverslips in 24-well plates, cells were then washed once with buffer and treated with vehicle (HBSS buffer), fibrinogen (2.6 mg/mL), FX (10 μg/mL), APC (300 nM), FXIa (20 μg/mL), FXIIa (20 μg/mL) in the presence of $ZnCl_2$ (25 μM) for 15 min at 37 °C. Cells were then washed again with HBSS buffer and fixed with 4% paraformaldehyde (PFA), then followed previously described antibody labeling as described in Section 2.3.

2.5. Image analysis

For presentation of data, the fluorescent intensities of each image were adjusted based on signals detected in neutrophil samples in the absence of primary antibodies. Quantification of fluorescent images was performed using a custom algorithm in MATLAB (The Mathworks, Inc., Natick, MA). This algorithm first establishes global thresholding parameters on training set fluorescence image data to then systematically quantify intensity profiles across treatment conditions. Quantitative comparison of treatment conditions was achieved by the normalization of fluorescence intensities to the number of cells per image to determine the percent and area of DNA and coagulation factor binding per cell.

3. Results

3.1. Quantification of coagulation factor binding to neutrophils during NETosis in a purified system

Activation of coagulation factors involved in thrombus formation, including fibrinogen and members of the intrinsic pathway of coagulation, have been reported to be associated with NETs formation in the context of immunothrombosis (Fuchs et al., 2010; Oehmcke et al., 2009). The overall goal was to design a platform to quantify the binding

Please cite this article as: Healy, L.D., et al., Colocalization of neutrophils, extracellular DNA and coagulation factors during NETosis: Development and utility of an immunofluor..., J. Immunol. Methods (2016), http://dx.doi.org/10.1016/j.jim.2016.06.002