Title: P-selectin promotes neutrophil extracellular trap formation in mice

Short title: P-selectin in NET formation

Julia Etulain,1,2 Kimberly Martinod,2,3,4 Siu Ling Wong,2,3 Stephen M. Cifuni2, Mirta Schattner,1 and Denisa D. Wagner2,3,5

1Laboratory of Experimental Thrombosis, Institute of Experimental Medicine, CONICET-National Academy of Medicine, Buenos Aires, Argentina. 2Program in Cellular and Molecular Medicine, Boston Children’s Hospital, Boston, Massachusetts, USA. 3Department of Pediatrics, Harvard Medical School, Boston, Massachusetts, USA. 4Immunology Graduate Program, Division of Medical Sciences, Harvard Medical School, Boston, MA, USA. 5Division of Hematology/Oncology, Boston Children’s Hospital, Boston, Massachusetts, USA.

Corresponding Author: Denisa D. Wagner, Ph.D. Program in Cellular and Molecular Medicine, Boston Children’s Hospital, 3 Blackfan Circle, Third Floor, Boston, MA 02115. USA

Email: Denisa.Wagner@childrens.harvard.edu

Phone: 1-617-713-8300

Fax: 1-617-713-8333

Word counts for text: 1439

Word counts for abstract: 170

Figure count: 2

Supplemental Figure: 1

Reference count: 27

Scientific category of submission: Brief Report
Key Points:
NET formation is stimulated by platelet or soluble P-selectin.

ABSTRACT
Neutrophil extracellular traps (NETs) are released in the vasculature. Besides trapping microbes, they promote inflammatory and thrombotic diseases. Considering that P-selectin induces prothrombotic and proinflammatory signaling, we studied the role of this selectin in NET formation. NET release (NETosis) was induced by thrombin-activated platelets rosetting with neutrophils and was inhibited by anti-P-selectin aptamer or anti-P-selectin glycoprotein ligand-1 (PSGL-1) inhibitory antibody, but not induced by platelets from P-selectin¬/¬ mice. Moreover, NETosis was also promoted by P-selectin-Ig fusion protein but not by control Ig. We isolated neutrophils from mice engineered to overproduce soluble P-selectin, the P-selectin∆CT/∆CT mice. While the levels of circulating DNA and nucleosomes (indicative of spontaneous NETosis) were normal in these mice, basal neutrophil histone citrullination and presence of P-selectin on circulating neutrophils were elevated. NET formation after stimulation with PAF, ionomycin, or PMA was significantly enhanced, indicating that the P-selectin∆CT/∆CT neutrophils were primed for NETosis. In summary, P-selectin, cellular or soluble, through binding to PSGL-1, promotes NETosis, suggesting that this pathway is a potential therapeutic target for NET-related diseases.
INTRODUCTION

Neutrophil extracellular traps (NETs) are extracellular DNA fibers comprising histones and neutrophil antimicrobial proteins. Although NETs trap pathogens and may play a beneficial role against infections, a growing body of evidence indicates that NETs are harmful in many inflammatory and thrombotic diseases including sepsis, coronary artery disease, and microvascular and deep vein thrombosis, highlighting NETs as a potential new target for therapeutic intervention. Platelets, through P-selectin, were shown to activate neutrophil integrins, and activated platelets were implicated in NETosis. Considering that platelet-neutrophil interactions involve cell-to-cell crosstalk mainly mediated by P-selectin, here we studied the role of P-selectin in NET formation (NETosis).

METHODS

For a full description of all methods, see Supplemental Materials and Methods available on the Blood website.

Animals

Eight- to ten-week old male or female mice, all on a C57BL/6J background, were used in the study. P-selectin$^{ΔCT/ΔCT}$ and P-selectin$^{-/-}$ mouse colonies were maintained in-house at Boston Children’s Hospital and were routinely crossed to the C57BL/6J background. Wild-type (WT) C57BL/6J mice were purchased from The Jackson Laboratory. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Boston Children’s Hospital (protocol number 14-03-2631R).

NET induction in vitro
Isolated peripheral blood neutrophils were allowed to adhere and incubated with thrombin-activated or resting platelets in the presence or absence of the P-selectin blocking aptamer ARC5690, anti-PSGL-1 blocking antibody (4RA10, BD Pharmingen) or the ROS inhibitor (TEMPOL), purified mouse P-selectin-Ig fusion protein (P-sel-Ig, BD Pharmingen), platelet activating factor (PAF, Calbiochem), ionomycin (Invitrogen), or phorbol 12-myristate 13-acetate (PMA, Sigma). Cells were stained with anti-citrullinated histone H3 (H3Cit, Abcam) and Hoechst-33342 (Invitrogen) and NETosis was analyzed by microscopy.

**Flow cytometry studies**

Isolated peripheral blood neutrophils were stained with allophycocyanin (APC)-conjugated anti-Ly6G and FITC-conjugated anti-P-selectin to detect P-selectin staining on neutrophils or APC-conjugated anti-Ly6G and Cell ROX Green to detect ROS generation by flow cytometry.

**NET determination in vivo**

Plasma levels of DNA (Quant-iT Picogreen dsDNA, Invitrogen) and nucleosomes (Cell Death Detection ELISA, Roche) were measured according to manufacturer’s instructions.

**Statistics**

Data were analyzed using GraphPad Prism software by two-sided Student t test or two-sided Mann–Whitney test performed between groups. Results were expressed as mean ± SEM and were considered significant at $P < 0.05$.

**RESULTS AND DISCUSSION**

P-selectin on activated platelets is required for platelet-mediated NET induction
To study the role of platelet P-selectin in NETosis, platelets were stimulated with thrombin and then overlaid on adherent neutrophils. While thrombin alone or unstimulated platelets failed to induce NETosis, neutrophil incubation with thrombin-stimulated platelets resulted in a significant increase in the percentage of both H3Cit+ cells and neutrophils releasing NETs (Figure 1A). NETosis mediated by activated WT platelets was completely inhibited by the P-selectin blocking aptamer, and not the control aptamer (Figure 1A). Platelets from P-selectin−/− mice did not induce H3Cit or NETosis (Figure 1B). Differential interference contrast (DIC) microscopy showed that while unstimulated WT platelets remained on the well bottom without interacting with each other or neutrophils, thrombin stimulation promoted WT platelet rosetting with the neutrophils (Figure 1C), a process known to be mediated by P-selectin.14 Although thrombin-stimulated P-selectin−/− platelets or WT platelets treated with the P-selectin aptamer formed homotypic aggregates, we confirmed that they did not interact with neutrophils (Figure 1C). In addition, blocking of neutrophil PSGL-1 completely inhibited the histone H3 citrullination induced by activated platelets, indicating that the signaling leading to histone citrullination was mediated by P-selectin-PSGL-1 interaction (Figure 1D).

**P-sel-Ig chimera or elevated plasma soluble P-selectin (sP-sel) promote NETosis**

To examine whether P-selectin is sufficient to stimulate NETosis, murine neutrophils were incubated *in vitro* with P-sel-Ig, a molecule previously shown to induce production of tissue factor-containing microparticles from monocytes.15 Recombinant P-selectin, was also shown to trigger protein tyrosine phosphorylation in neutrophils and the tyrosine kinase-dependent function of Mac-1.16,17 A significant increase in the percentage of H3Cit+...
neutrophils and cells forming NETs was observed after the addition of the P-sel-Ig to WT neutrophils while control Ig had no such effects (Figure 1E). Although both activated platelets and P-sel-Ig induced NETosis, our results indicate that activated platelets were more potent stimulants than P-sel-Ig. This could be due to differences in the local concentration achieved and quality of the P-selectin presented to the neutrophils in these experiments. Alternatively, it is possible that once the signaling through P-selectin/PSGL-1 is established, platelets may provide other co-stimulatory signals to the neutrophils leading to enhanced NETosis. These could be receptor mediated such as through binding of platelet GPIbα to activated αMβ2 or by soluble factors released by the platelets interacting with the neutrophils.

To study whether in vivo released sP-sel in mice may also have a stimulatory effect on circulating neutrophils, we evaluated P-selectin ΔCT/ΔCT mice that have five-fold elevated plasma sP-sel. These mice express P-selectin without the cytoplasmic domain leading to proteolytic shedding of P-selectin from endothelium. To evaluate NETosis, we first examined plasma DNA and nucleosome levels in these mice and found that they were similar to those of WT (DNA WT=92±10 vs. P-selectin ΔCT/ΔCT =84±2 (ng/ml), p=0.43; nucleosomes WT=0.05±0.01 vs. P-selectin ΔCT/ΔCT =0.042±0.01 (Absorbance 405nm), p=0.68) indicating that at steady state, in either genotype, NETs do not appear to be spontaneously released into the circulation. However, knowing that the P-selectin ΔCT/ΔCT mice have a worse phenotype than WT mice in many pathological conditions known to produce NETs, we were wondering whether their neutrophils could be primed for NETosis. We isolated neutrophils from WT and P-selectin ΔCT/ΔCT mice and incubated them in the absence or presence of different stimulants for NETosis (Figure 2). Indeed, even
without stimulation the levels of H3Cit+ neutrophils were higher when obtained from the P-selectin$^{ΔCT/ΔCT}$ mice, indicating that the chromatin modification leading to NETosis occurred more frequently in the P-selectin$^{ΔCT/ΔCT}$ neutrophils than in WT (Figures 2A-D), although neither genotype produced NETs without additional stimulation (Figures 2A-D). The percentage of cells forming NETs upon neutrophil stimulation by PAF (Figure 2A), ionomycin (Figure 2B), or PMA (Figure 2C) were all significantly higher in neutrophils from P-selectin$^{ΔCT/ΔCT}$ compared to WT mice, confirming that neutrophils from the P-selectin$^{ΔCT/ΔCT}$ mice are primed to release NETs. Furthermore, the circulating neutrophils of P-selectin$^{ΔCT/ΔCT}$ mice had P-selectin bound to their surface but not the neutrophils from WT or control P-selectin$^{-/-}$ mice (Figure 2E), suggesting that it could be constitutively priming these neutrophils. Hazeldine et al. recently concluded that the induction of ROS generation is one of the mechanisms by which TNF-α primes neutrophils.$^{21}$ We also were able to show that P-selectin promotes ROS generation in neutrophils, and that NET formation induced by this selectin is completely blocked by the pharmacologic inhibition of ROS by TEMPOL (Supplemental Figure 1). Our results based on the P-sel-Ig treatment and the genetically engineered mice overexpressing sP-sel indicate that sP-sel is the priming factor in the P-selectin$^{ΔCT/ΔCT}$ mice, however, we do not rule out that other circulating inflammatory factors such as the monocyte-derived pro-coagulant microparticles$^{22}$ may also contribute to the neutrophil priming. Our data suggest that increased levels of sP-sel, seen in many chronic thrombotic and inflammatory conditions, may sensitize neutrophils for NETosis which may further aggravate the pathology. Indeed the increase in sP-sel in P-selectin$^{ΔCT/ΔCT}$ mice is associated with a pro-coagulant and pro-inflammatory phenotype$^{19,22}$ that results in larger thrombi in a deep vein thrombosis model,$^{20}$ more extensive infarcts in the middle cerebral
artery ischemia/reperfusion stroke model,\textsuperscript{19} and increased susceptibility to atherosclerosis on an ApoE\textsuperscript{−/−} background.\textsuperscript{19} We demonstrate in this study that sP-sel is also priming neutrophils for NETosis, a new mechanism likely contributing to the phenotype of the P-selectin\textsuperscript{ΔCT/ΔCT} mice. Our finding supports the notion that sP-sel is an important biomarker predictive of future thrombotic and inflammatory events.

Activated platelets are known to have many stimulating effects upon their adhesion to leukocytes on the leukocytes themselves and even surrounding cell types, such as endothelium.\textsuperscript{9} Recently, activated platelets were shown to enhance NETosis in mouse models such as acute lung injury\textsuperscript{8} or sepsis.\textsuperscript{23} In one study the high mobility group box 1 presented by activated platelets to the neutrophils was implicated in the NETotic process.\textsuperscript{24} P-selectin is the most important molecule mediating platelet-leukocyte interaction triggering activating signaling into leukocytes primarily through PSGL-1. We now show that platelet P-selectin primes neutrophils for NETosis. It is likely that E-selectin expressed by inflamed endothelium that also binds to PSGL-1\textsuperscript{9} has the same effect. Interestingly, PSGL-1 blockade was recently shown to reduce NET biomarkers (MPO-DNA) in plasma in acute lung inflammation and sepsis models.\textsuperscript{25}

Although the relevance of our experimental data in mice remains to be determined in clinical settings, increased levels of sP-sel and platelet-leukocyte complexes have been reported in many human diseases.\textsuperscript{26,27} The present finding that P-selectin promotes NETosis further supports the clinical development of P-selectin/PSGL-1 inhibitors as therapeutic agents to reduce pathological thrombosis and inflammation.
ACKNOWLEDGEMENTS

The authors thank Melanie Demers for helpful discussions. The P-selectin inhibitory and control aptamers were generously provided by Dr. Robert Schaub (Archemix, Cambridge, MA). This work was supported by the National Heart, Lung, and Blood Institute of the National Institutes of Health Grant R01HL102101 to D.D.W., and J.E. was the recipient of a Reach the World Fellowship from the International Society on Thrombosis and Haemostasis.

AUTHORSHIP CONTRIBUTIONS

J.E. performed and designed research, performed the experiments, analyzed and interpreted data, and wrote the manuscript. K.M. obtained preliminary results on P-selectin$^\Delta\mathrm{CT}/\Delta\mathrm{CT}$ mice, performed the DNA and nucleosome experiments, P-selectin staining on neutrophils and NETosis mediated by platelets in the presence of anti-PSGL-1 blocking antibody. S.L.W. and S.M.C. taught and assisted J.E. with the experimental methodology. K.M., S.L.W., M.S., and D.D.W. helped to analyze and interpret results. M.S. contributed to writing the manuscript. D.D.W. designed the objectives of the work, provided a critical and substantive review of the intellectual work, and co-wrote the manuscript.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.
REFERENCES


FIGURE LEGENDS

Figure 1. P-selectin is a stimulant of NET formation. A) NET formation was induced by unstimulated- or thrombin-stimulated platelets in the presence of P-selectin aptamer inhibitor ARC5690 (P-sel ap) or ARC5694 as control aptamer (Control ap). NET formation in medium with or without thrombin was also evaluated (n = 5). B) NET formation was induced by unstimulated- or thrombin-stimulated platelets from WT or P-selectin \(^{-/-}\) mice (P-sel\(^{-/-}\)) (n = 5). C) The behavior of platelets in experiments A and B was visualized by DIC microscopy. The images are representative from 5 experiments (scale bar 5 µm). D) NET formation was examined after incubation with unstimulated- or thrombin-stimulated WT platelets in the presence of anti-PSGL-1 blocking antibody or its Ig control. The percent of H3Cit+ neutrophils was quantified (n = 4, *P < 0.05). E) Left: WT neutrophils were stimulated with P-sel-Ig or with the same class of immunoglobulin as a control (Ig) (n = 4, **P < 0.01; ***P < 0.001). Right: Representative fluorescence microscopy images of control-Ig or P-sel-Ig treatments showing H3Cit negative neutrophils (yellow arrow), H3Cit positive neutrophils without NET release (red arrow), and H3Cit positive neutrophils releasing NETs (white arrowhead) (scale bar 20 µm).

Figure 2. Neutrophils of P-selectin\(^{ACT/ACT}\) mice are primed to release NETs in vitro. Neutrophils isolated from peripheral blood of WT and P-selectin\(^{ACT/ACT}\) (ΔCT/ΔCT) mice were kept unstimulated (Unst) or stimulated with PAF (A), ionomycin (iono) (B), or PMA (C). The percentage of H3Cit+ neutrophils and NET release were quantified (n = 4, *P < 0.05; **P < 0.01; ***P < 0.001). D) Representative fluorescence microscopy images of unstimulated- or ionomycin-stimulated cells showing H3Cit negative neutrophils (yellow
arrow), H3Cit positive neutrophils without NET release (red arrow), and H3Cit positive neutrophils releasing NETs (white arrowhead) (scale bar 20 µm). E) Neutrophils isolated from peripheral blood of P-selectin^−/−, WT or P-selectin^{ΔCT/ΔCT} (ΔCT/ΔCT) mice were double stained with APC-conjugated anti-Ly6G and P-selectin-FITC and the mean fluorescence intensity (MFI) of P-selectin-positive cells were analyzed in the neutrophil gate (Ly6G-positive cells) by flow cytometry (n = 4-5, *P < 0.05). Representative FACS plots for each genotype are shown on the right.
Figure 1

A and B: Graphs showing the percentage of H3Cit+ cells in different conditions.

C: Images comparing neutrophils and platelets with and without platelets and thrombin.

D and E: Graphs showing the percentage of H3Cit+ cells and NET release under different conditions.
P-selectin promotes neutrophil extracellular trap formation in mice

Julia Etulain, Kimberly Martinod, Siu Ling Wong, Stephen M. Cifuni, Mirta Schattner and Denisa D. Wagner