ORIGINAL ARTICLE

Histones link inflammation and thrombosis through the induction of Weibel–Palade body exocytosis

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Essentials

- Dysregulated DNA and histone release can promote pathological immunothrombosis.
- Weibel–Palade bodies (WPBs) are sentinel-like organelles that respond to proinflammatory stimuli.
- Histones induce WPB exocytosis in a caspase, calcium and charge-dependent mechanism.
- A targetable axis may exist between DNA/histones and WPBs in inflammation and immunothrombosis.

Summary. *Background:* Damage-associated molecular patterns (DAMPs), including molecules such as DNA and histones, are released into the blood following cell death. DAMPs promote a procoagulant phenotype through enhancement of thrombin generation and platelet activation, thereby contributing to immunothrombosis. Weibel–Palade bodies (WPBs) are dynamic endothelial cell organelles that contain procoagulant and proinflammatory mediators, such as von Willebrand factor (VWF), and are released in response to cell stresses. VWF mediates platelet adhesion and aggregation, and has been implicated as a procoagulant component of the innate immune response. *Objective:* To determine the influence of histones and DNA on WPB release, and characterize their association in models of inflammation. *Methods:* We treated C57BL/6J mice and cultured endothelial cells with histones (unfractionated, lysine-rich or arginine-rich)

and DNA, and measured WPB exocytosis. We used inhibitors to determine a mechanism of histone-induced WPB release *in vitro*. We characterized the release of DAMPs and WPBs in response to acute and chronic inflammation in human and murine models. *Results and conclusions:* Histones, but not DNA, induced the release of VWF (1.46-fold) from WPBs and caused thrombocytopenia (0.74-fold), which impaired arterial thrombus formation in mice. Histones induced WPB release from endothelial cells in a caspase-dependent, calcium-dependent and charge-dependent manner, and promoted platelet capture in a flow chamber model of VWF–platelet string formation. The levels of DAMPs and WPB-released proteins were elevated during inflammation, and were positively correlated in chronic inflammation. These studies showed that DAMPs can regulate the function and level of VWF by inducing its release from endothelial WPBs. This DAMP–WPB axis may propagate immunothrombosis associated with inflammation.

Keywords: endothelial cells; histones; inflammation; von Willebrand factor; Weibel–Palade bodies.

Introduction

Immunothrombosis is a recently introduced term that describes the interaction between inflammatory mediators and the coagulation system [1]. Physiologic immunothrombosis may provide a scaffold for the localized concentration of antimicrobial proteins, the sequestration of pathogens, and the facilitation of innate immune cell recruitment [1].

In contrast, dysregulated immunothrombotic processes can result in uncontrolled coagulation activation, leading to pathologic thrombus formation [2,3]. Inflammation can cause tissue stress, leading to the release of damage-associated molecular patterns (DAMPs). DAMPs

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constitute a group of endogenous, intracellular molecules that includes DNA, RNA, histones, and ATP [4]. DAMPs bind to pattern-recognition receptors, such as Toll-like receptors (TLRs), present on platelets, and innate immune and endothelial cells, and activate downstream innate inflammatory pathways [5]. Additionally, DAMPs have been shown to regulate coagulation activation in immunothrombosis [1]. Neutrophil extracellular traps (NETs) constitute a source of DAMPs, including DNA, histones, and proteolytic enzymes, that localize pathogen infection and have been shown to mediate contact pathway activation [6], contribute to platelet activation [7–9], modulate fibrinolysis [10], and increase tissue factor availability [11].

Endothelial cells are regulators of coagulation activation in immunothrombosis. Under physiologic conditions, endothelial cells maintain blood in its fluid state by regulating the secretion of procoagulant, proinflammatory and proangiogenic factors, including von Willebrand factor (VWF), P-selectin, interleukin-8, and angiopoietin-2 (ANG-2), by packaging them in intracellular storage granules termed Weibel–Palade bodies (WPBs). Upon exposure to proinflammatory or proapoptotic stimuli (such as thrombin [12] or hypoxia [13]), the endothelium can rapidly upregulate a procoagulant phenotype by releasing WPB contents.

VWF is a multimeric glycoprotein that mediates platelet adhesion and aggregation at sites of endothelial damage, and acts as a carrier protein for coagulation factor VIII [14]. VWF is also stored in platelet α -granules, and is released to provide the scaffold needed for fibrin deposition. Additionally, VWF promotes leukocyte recruitment and extravasation under conditions of inflammation [15]. Plasma levels of VWF constitute a key regulator of thrombosis, whereby elevated VWF levels are associated with an increased risk of venous [16] and arterial [17] thrombosis.

Previous studies have suggested a relationship between the release of DAMPs and VWF activity and antigen levels. Fuchs *et al.* demonstrated that NETs can capture platelets and VWF under conditions of shear [3], which may involve histone binding to the VWF A1 domain [18]. Exposure of isolated platelets to histones induces platelet activation and VWF release [19], and infusion of unfractionated histones into mice induces severe thrombocytopenia [20] and increases VWF antigen (VWF:Ag) levels [21]. However, in these studies, the mechanism(s) by which histones modulate VWF:Ag levels and the contribution of endothelial cells to this increase were not investigated. Moreover, the influence of DAMPs on other proinflammatory and procoagulant agents that may be secreted along with VWF has not been characterized.

We hypothesized that exposure of endothelial cells to DAMPs can mediate WPB exocytosis, thereby increasing plasma levels of VWF and WPB contents. We have therefore mechanistically described WPB exocytosis in response

to DNA and histones, and examined the association between WPB contents and DAMPs in inflammation.

Materials and methods

Reagents

The following reagents were used: calf thymus unfractionated histone (UH) (Worthington Biochemical Corporation, Lakewood, NJ, USA); calf thymus lysine-rich (HK) and arginine-rich (HR) histone fractions, calf thymus DNA, phorbol 12-myristate 13-acetate (PMA), protamine sulfate, apyrase, rhodamine 6G, hematoporphyrin, staurosporine, and lipopolysaccharide (LPS) (Sigma-Aldrich, St Louis, MO, USA); pan-caspase inhibitor (Z-VAD-FMK; R&D Systems, Minneapolis, MN, USA); BAPTA-AM and prostacyclin (Santa Cruz, Dallas, TX, USA); anti-TLR-2 (clone T2.5), anti-TLR-4 (HTA125), and annexin V (eBioscience, San Diego, CA, USA); and 3,3'-dihexyloxacarbocyanine iodide (DiOC6) (Invitrogen, Burlington, Ontario, Canada).

Histones

There are five classes of histones that function together to bind DNA, including lysine-rich HK histone H1, slightly HK histones H2a and H2b, and arginine-rich (HR) histones H3 and H4. We used calf thymus HK and HR histone fractions to analyze their differential effects on WPB release. Although HK and HR preparations are composed mainly of histones H1 and H3/4, respectively, these preparations are not 100% pure. To justify the use of bovine products in murine and human models, we performed an alignment search with the NCBI BLAST online tool; the results of this are summarized in Table S1.

DNA and histone injections into mice

Normal C57BL/6J mice (aged 9–11 weeks) received retro-orbital injections of histones ($10\text{--}40\text{ mg kg}^{-1}$) [21,22] or DNA (4 mg kg^{-1}). One hour after treatment, blood was collected from the inferior vena cava into 10% buffered citrate. Platelets were quantified by use of a complete blood count (ABC Hematology Analyzer; scil Vet, Gurnee, IL, USA). Plasma concentrations were normalized to a blood sample obtained 1 week prior to the experiment to limit intermouse variability. All mouse experiments were approved by the Queen's University Animal Care Committee.

Murine intravital model of arterial thrombosis

An intravital model of arterial thrombosis as described by Nishimura *et al.* was used [23]. Mice received saline or UH (20 mg kg^{-1}) immediately prior to jugular vein cannulation and exteriorization of the cremaster muscle.

Endogenous platelets were labeled with DiOC6 (0.12 μM). Hematoporphyrin was administered at 3 mg kg^{-1} prior to the first injury, and at 1 mg kg^{-1} for each subsequent injury. The vessel was imaged with a Quorum WaveFX-X1 spinning disk microscope (Quorum Technologies, Guelph, ON, Canada) at a wavelength of 488 nm for 6.67 min. A thrombus was considered to be occlusive when it reached both sides of the vessel wall or caused cessation of blood flow. The imaging technician was blinded to the experimental conditions.

Murine platelets were isolated as described by Darbousset *et al.* [24], and were labeled with rhodamine 6G at 1 $\mu\text{g mL}^{-1}$. Each mouse received a bolus of $\sim 3 \times 10^6$ platelets after a baseline injury had been performed in a histone-treated mouse. Mice received $\sim 1 \times 10^6$ platelets before each subsequent injury.

Static endothelial cell experiments

Blood outgrowth endothelial cells (BOECs) were isolated as previously described [25,26]. Human umbilical cord vein endothelial cells (HUVECs) were purchased from Lonza (Mississauga, Ontario, Canada). BOECs (passages 5–15) and HUVECs (2–5 passages) were seeded (1×10^6 cells mL^{-1}) onto collagen-coated plates for 24 h. Cells were stimulated with PMA (100 nM), UH, HK histone fraction, HR histone fraction (5–25 $\mu\text{g mL}^{-1}$), DNA (25 $\mu\text{g mL}^{-1}$) or DNA/histone combinations (each 25 $\mu\text{g mL}^{-1}$) in serum-free medium (Opti-MEM; Life Technologies, Grand Island, NY, USA) for 2 h at 37 °C.

In mechanistic studies, cells were pretreated for 1 h at 37°C with selective antagonists: BAPTA-AM (10 μM), pan-caspase inhibitor (Z-FMK-VAD, 100 μM), protamine sulfate (150 $\mu\text{g mL}^{-1}$), anti-TLR-2/4 antibodies (20 $\mu\text{g mL}^{-1}$), or annexin V (20 $\mu\text{g mL}^{-1}$). Cells were subsequently stimulated with PMA (100 nM), HK histone fraction (12.5 $\mu\text{g mL}^{-1}$) or staurosporine (200 nM) for 2 h. The medium was analyzed for VWF:Ag, ANG-2 and P-selectin levels.

Propidium iodide staining

BOECs were treated with HK histone fraction (12.5 $\mu\text{g mL}^{-1}$) for 2 h. Cells were incubated with propidium iodide (10 $\mu\text{g mL}^{-1}$) for 10 min at room temperature. Cells were then trypsinized (0.25% trypsin–EDTA) and subjected to flow cytometry with a MACSQuant Analyzer 10 (Miltenyi Biotec, Bergisch Gladbach, Germany).

Visualization of VWF–platelet strings in a flow chamber model

Washed platelets were obtained from healthy volunteers [27], and labeled with 1 μM DiOC6. BOECs were seeded onto collagen-coated flow chambers (ibidi III^{0.1}; ibidi,

Munich, Germany) and stimulated with histamine (25 nM), PMA (100 nM), UH, HK histone fraction or HR histone fraction (25 $\mu\text{g mL}^{-1}$) for 15 min. Platelets were perfused over the stimulated BOECs at a shear rate of 500 s^{-1} for 10 min. Images were taken during and after flow with a $\times 20$ objective lens on the Quorum WaveFX-X1 spinning disk confocal system, and microscope control and data acquisition/analysis were performed with METAMORPH Microscopy Automation and Image Analysis Software (Molecular Devices, Sunnyvale, CA, USA); the technician was blinded. VWF–platelet strings (three or more aligned platelets) were quantified from post-flow still images of 10 consecutive fields of view.

Human models of chronic and acute inflammation

Subjects with chronic inflammation (aging) were from a previously collected cohort [28], and were classified as young (mean age 7 ± 5 years, range 1–17 years, $n = 51$), middle-aged (mean age 41 ± 6 years, range 30–49 years, $n = 42$) and old (mean age 71 ± 7 years, range 55–87 years, $n = 67$) groups. All groups were similar in gender and ABO blood type composition. These studies were approved by the Research Ethics Board of Queen's University, Canada.

Subjects with acute inflammation (sepsis) were collected as part of the ongoing DNA as a Prognostic Marker in ICU Patients Study (DYNAMICS Study) within 24 h of enrollment, were not treated for a thrombotic episode, and had few comorbidities [29]. Additional information can be found in Table S2. These studies were approved by the Research Ethics Board of McMaster University and Hamilton Health Sciences, Canada.

Mouse models of chronic and acute inflammation

Blood samples from 9-week-old and 55-week-old normal C57BL/6J mice were obtained by cardiac puncture. In a mouse model of endotoxemia, C57BL/6J mice received a 5 mg kg^{-1} intraperitoneal injection of LPS. Blood samples were collected from the retro-orbital plexus at 3 h, 6 h, 9 h and 12 h after injection.

Biomarker quantification

Human and mouse VWF:Ag levels were quantified from plasma and cell supernatants by ELISA with polyclonal anti-VWF antibodies (A0082 and P0026; Dako, Glostrup, Denmark) [30]. Murine and human ANG-2 levels were quantified from plasma and cell supernatants by ELISA (respectively: Quantikine ELISA and DuoSet ELISA; R&D Systems, Minneapolis, MN, USA). P-selectin was quantified from cell supernatants by ELISA (DuoSet ELISA). Cell-free DNA (CF-DNA) was quantified from plasma with the Quant-iT PicoGreen assay (Invitrogen) [31].

Statistical analyses

Statistical analysis was performed with GRAPHPAD PRISM 4.03 for Windows (GraphPad Prism, San Diego, CA, USA). Data are presented as mean values $\pm$ standard errors. Significance of differences was determined with Mann–Whitney tests. Linear regression analysis was performed for associations of age and VWF:Ag, and of age and CF-DNA. Spearman non-parametric correlation testing was used for correlation analyses between CF-DNA and WPB constituents.

Results

Histones induce WPB release and thrombocytopenia in mice

Previous studies by Fuchs *et al.* suggested that intravenous administration of UH induced severe

thrombocytopenia [20] and also increased VWF:Ag levels (by $\sim 21\%$) after 1 h [21]. We first confirmed that, 1 h after injection, UH induced systemic VWF release in a dose-dependent manner ($10\text{--}40\text{ mg kg}^{-1}$, $1.22\text{--}1.46$ fold, $P = 0.042\text{--}0.001$) relative to saline-injected controls (Fig. 1A). We further demonstrated that both HK (1.61-fold, $P = 0.004$) and HR (1.22-fold, $P = 0.046$) histone fractions (20 mg kg^{-1}) increased VWF:Ag levels. Injection of histone-free DNA (which generated a CF-DNA plasma level equivalent to that in a murine model of endotoxemia [$\sim 4000\text{ ng mL}^{-1}$; Fig. 7D]) was not associated with an increase in VWF:Ag levels. ANG-2 levels were also elevated in all mice treated with histones (Fig. 1B), suggesting that the histone-induced release of VWF is, at least in part, from endothelial WBPs.

The histone-induced increase in VWF:Ag levels may also be related to platelet activation and α -granule secretion. We confirmed that UH and HK histone fractions

Fig. 1. Histones induce Weibel–Palade body release and thrombocytopenia *in vivo*. Normal C57BL/6J mice received retro-orbital injections of unfractionated histone (UH), arginine-rich (HR) or lysine-rich (HK) histone fraction, DNA, or saline (control [Ctrl]). von Willebrand factor antigen (VWF:Ag) (A) and angiopoietin-2 (ANG-2) (B) levels and platelet count (C) were measured. Data are shown as mean values $\pm$ standard error ($n \geq 7$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

induced thrombocytopenia in mice (Fig. 1C). HK histone fraction induced a comparable reduction in platelet count (0.78-fold, $P = 0.0068$) to that observed with UH (0.74-fold, $P = 0.0005$). HR histone fraction (0.89-fold, $P = 0.10$) or DNA (1.06-fold, $P = 0.65$) did not significantly modify the platelet count as compared with saline. We also showed that VWF is not required for histone-induced thrombocytopenia in VWF-deficient mice (Fig. S1), suggesting that histones may directly interact with platelets *in vivo*.

To investigate the systemic consequence of exposure to histone on thrombus formation, we utilized a previously described hematoporphyrin-induced model of arterial thrombosis [23]. UH was administered 1 h prior to the induction of thrombosis. Thrombus formation was significantly impaired in UH-treated mice (Fig. 2D–F) as compared with saline controls (Fig. 2A–C; $P < 0.0001$). Infusion of isolated murine platelets immediately prior to thrombus initiation restored the ability of UH-treated mice to form a stable occlusion (Fig. 2G–I). The fact that this defect in thrombogenesis can be rescued by normal

platelet infusion indicates that histone-induced thrombocytopenia or impaired platelet function has a significant influence on the hemostatic balance in circumstances of DAMP release.

Histones induce WPB release from human endothelial cells

To directly assess the influence of histones and DNA on VWF release from endothelial cells, we exposed BOECs to PMA (positive control), UH, HR histone fraction, HK histone fraction, DNA and DNA/histone combinations for 2 h. We observed that HK histone fraction ($5\text{--}25\text{ }\mu\text{g mL}^{-1}$) induced VWF and ANG-2 release from BOECs in a dose-dependent manner (~ 2 -fold; Fig. 3A, B). Similarly, more soluble P-selectin was detected in the PMA-treated and HK histone fraction-treated BOEC medium (Fig. 3C) [32]. Interestingly, when BOECs were stimulated with UH and HR histone fraction, we observed less VWF:Ag in the medium than in the untreated condition (Fig. S2A). We also showed that HR histone fraction and UH bound more strongly to VWF

Fig. 2. Arterial thrombus formation is impaired by histone administration but can be restored following platelet infusion. Thrombus formation was determined in normal C57Bl/6J mice treated with unfractionated histone (UH) or saline (Untreated). Endogenous platelets were labeled with 3,3'-dihexyloxacarbocyanine iodide (green), and infused platelets were labeled with rhodamine (blue). Representative images of thrombus development from saline-treated mice (A–C), UH-treated mice (D–F), and UH-treated mice infused with exogenous platelets (UH + platelets) (G–I). Time to occlusion was measured for each injury. $n \geq 4$ mice with $n \geq 3$ injuries per mouse (J). * $P < 0.05$ and *** $P < 0.001$.

than HK histone fraction, suggesting that histone–VWF interactions could affect the quantification of VWF:Ag by ELISA in this model (Fig. S2B,C). To confirm that HR histone fraction and UH facilitated WPB exocytosis, we demonstrated that exposure to histones induced ANG-2 and/or P-selectin release from endothelial cells.

Histones mediate VWF–platelet string formation in a flow chamber model

As DAMPs can induce both VWF release from endothelial cells and regulate platelet activation, we used a model of VWF–platelet string formation [33] to investigate the effect of histone exposure on VWF–platelet interactions (Videos S1a,b).

We observed that, following initiation of flow conditions, untreated cells had very few VWF–platelet strings

(< 1 string per review field), whereas histamine-treated and PMA-treated cells had an average of 2.10 and 3.10 strings, respectively. Significantly more VWF–platelet strings were formed when cells were treated with HK histone fraction (3.52 strings, $P = 0.019$), HR histone fraction (6.00 strings, $P = 0.004$), and UH (5.51 strings, $P = 0.012$) (Fig. 4).

Histones induce WPB exocytosis by a caspase-dependent, calcium-dependent and charge-dependent mechanism

Previous studies by Xu *et al.* have shown that extracellular histones are cytotoxic to endothelial cells ($50 \mu\text{g mL}^{-1}$, 1 h) [22] and are associated with increased intracellular calcium levels [34]. We confirmed that the low level of HK histone fraction ($12.5 \mu\text{g mL}^{-1}$, 2 h) used to induce WPB secretion induced modest propidium iodide uptake

Fig. 3. Lysine-rich (HK) histones induce Weibel–Palade body (WPB) exocytosis from endothelial cells *in vitro*. Blood outgrowth endothelial cells were incubated with phorbol 12-myristate 13-acetate (PMA; positive control) or HK histone fraction for 2 h, and WPB contents were quantified in the medium. HK histone fraction induced dose-dependent release of von Willebrand factor antigen (VWF:Ag) (A), angiopoietin-2 (ANG-2) (B) and P-selectin (C) relative to untreated controls. Data are shown as mean values $\pm$ standard error. $n \geq 3$. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

Fig. 4. Histones mediate von Willebrand factor (VWF)–platelet string formation. Endothelial cells seeded on flow chamber slides were treated with unfractionated histone (UH), lysine-rich (HK) or arginine-rich (HR) histone fraction, or histamine (positive control), and perfused at a shear rate of 500 s^{-1} for 10 min with washed, fluorescently labeled platelets. Representative images were obtained after platelet flow from the untreated control (A) and cells treated with histamine (B), HK histone fraction (C), HR histone fraction (D), and UH (E). (F) Phorbol 12-myristate 13-acetate treatment resulted in an average of 3.1 strings per field of view, which was comparable to the results of histamine treatment. VWF–platelet strings were quantified from 10 consecutive review fields, and averaged for $n \geq 3$ individual experiments. Data are shown as mean values $\pm$ standard error. * $P < 0.05$ and ** $P < 0.01$ indicate significance relative to the untreated condition.

(Fig. 5A). Interestingly, increases in intracellular calcium levels have previously been demonstrated to facilitate WBP exocytosis [35]. We demonstrate that inhibition of apoptosis with the pan-caspase inhibitor (Z-VAD-FMK) (Fig. 5B) and calcium chelation with BAPTA-AM (Fig. 5C) inhibited HK histone fraction-induced ANG-2 release from BOECs ($P = 0.019$).

Studies have also suggested that histones interact directly with anionic phospholipids on the endothelial surface [34]. Preincubating cells with protamine sulfate (a polymer with a high positive charge density) or annexin V, which binds anionic phospholipids on the cell surface, inhibited the release of ANG-2 from BOECs in response to HK histone fraction stimulation ($P = 0.01$) (Fig. 5D,E). TLR-2 and TLR-4 could not be identified on the surfaces of BOECs (Fig. S3), so preincubation of BOECs with TLR-2/4 inhibitory antibodies was not protective against the release of WPBs (Fig. 5F). Studies were performed on HUVECs (Fig. S4A–C), and these showed similar trends to those seen with BOECs.

VWF:Ag and DAMP levels are correlated in human and animal models of chronic inflammation

We next evaluated the association between VWF:Ag and DAMP levels in models of acute (sepsis) and chronic (aging) inflammation. We used CF-DNA levels as a surrogate measure of DAMP release. In aging individuals, we observed that VWF:Ag ($\beta = 0.010$, 95% confidence interval [CI] 0.007–0.012, $P < 0.0001$) and CF-DNA ($\beta = 0.987$, 95% CI 0.287–1.687, $P = 0.006$) levels were significantly associated with age (Fig. 6A,B).

To investigate whether plasma levels of VWF were associated with CF-DNA levels, we performed correlative analyses, and found that, in the young and middle-age populations, there was no association between VWF and CF-DNA (Fig. S5A,B). However, we noted a significant, positive correlation between CF-DNA and WPB contents (VWF:Ag and ANG-2) in our old population (VWF:Ag, $r = 0.24$, $P = 0.036$; ANG-2, $r = 0.24$, $P = 0.048$) (Fig. 6B).

Fig. 5. Lysine-rich (HK) histones induce Weibel-Palade body (WPB) exocytosis by a caspase-dependent, calcium-dependent and charge-dependent mechanism. HK histone fraction (25 $\mu\text{g mL}^{-1}$) induced propidium iodide (PI) uptake (A). Blood outgrowth endothelial cells were pre-treated for 1 h with selective antagonists, and then stimulated with phorbol 12-myristate 13-acetate (PMA), HK histone fraction (12.5 $\mu\text{g mL}^{-1}$), or staurosporine (SS) (200 nM) for 2 h. HK histone fraction-mediated WPB exocytosis was impaired by inhibition of caspase activation with a pan-caspase inhibitor (Z-VAD-FMK) (B), calcium chelation with BAPTA-AM (C), and masking of negatively charged cell surface residues with protamine sulfate (D) or annexin V (E), but not by Toll-like receptor (TLR)-2/4 inhibition (F). Data are shown as mean values $\pm$ standard error. $n \geq 3$. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. ANG-2, angiopoietin-2; NS, not significant.

We similarly observed that CF-DNA and VWF:Ag levels were significantly higher in 55-week-old mice than in young (9-week-old) mice (VWF:Ag, 2.41-fold, $P < 0.0001$; CF-DNA, 1.33-fold, $P < 0.0001$) (Fig. 6C). Analysis of the 9-week-old and 55-week-old populations demonstrated a significant correlation between VWF and CF-DNA levels ($r = 0.78$, $P < 0.0001$) (Fig. 6D).

We next evaluated the association between plasma VWF:Ag and CF-DNA in acute inflammation. A cohort of 50 individuals diagnosed with severe sepsis had significantly higher levels of CF-DNA, VWF:Ag and ANG-2 than age-matched controls (Fig. 7A). The levels of CF-DNA and VWF, and of CF-DNA and ANG-2, were not correlated in this model (Fig. 7B). We also assessed the association between CF-DNA and VWF:Ag in a murine model of endotoxemia. We observed that VWF:Ag levels increased rapidly in the acute phase of this inflammatory model, and then declined to baseline levels after 12 h (Fig. 7C). In contrast, CF-DNA levels continued to exponentially increase throughout the 12 h period (Fig. 7D).

Therefore, plasma VWF and CF-DNA levels were not correlated in this model ($r = 0.12$, $P = 0.54$).

It has been previously demonstrated that, in addition to regulation of coagulation, VWF may also function as a regulator of inflammatory processes [15,36]. We addressed the influence of VWF on DAMP release, and found no association (Fig. S6A–C).

Discussion

WPBs are endothelial cell sentinels containing procoagulant and proinflammatory molecules that have the potential to regulate immunothrombosis. One candidate, VWF, facilitates platelet capture and the recruitment and extravasation of leukocytes [15] under inflammatory conditions. Similarly, endothelial P-selectin is essential for neutrophil diapedesis [37], and ANG-2 has been linked to increased vascular permeability in inflammatory disease [38]. Thus, WPBs serve as an important link between hemostatic and innate immune responses.

Fig. 6. Cell-free DNA (CF-DNA) and Weibel–Palade body contents are positively correlated in chronic inflammation associated with aging. Both von Willebrand factor antigen (VWF:Ag) ($\beta = 0.010$, 95% confidence interval [CI] 0.007–0.012, $P < 0.0001$) (A) and CF-DNA levels ($\beta = 0.987$, 95% CI = 0.287–1.687, $P = 0.006$) (B) were associated with age (linear regressions with 95% CIs [dotted lines] are shown). (C) Correlation between CF-DNA and VWF:Ag ($r = 0.24$, $P = 0.036$) and angiotensin-2 (ANG-2) ($r = 0.24$, $P = 0.048$). VWF:Ag and CF-DNA levels in old (55 weeks, $n = 14$) as compared with young (9 weeks, $n = 15$) mice (D). Correlation of CF-DNA and VWF in mice over an age continuum ($r = 0.69$, $P < 0.0001$) (E). O, old; Y, young. Data are shown as mean values $\pm$ standard error. *** $P < 0.001$.

Previous studies have suggested that UH can induce platelet activation [8,20] and VWF release from α -granules [19] – a potential source of the elevated VWF:Ag levels in our *in vivo* experiments and those performed by Brill *et al.* [21]. We extend these studies to demonstrate that all histone fractions induced significant VWF and ANG-2 release. This suggests that the increase in VWF:Ag levels may be attributable to both platelet activation and endothelial WPB exocytosis, although it is unknown what proportion of the increase in plasma VWF levels is attributable to either source.

We next assessed the ability of histones to induce the exocytosis of WPB *in vitro*. We observed that HK histone fraction induced WPB release (VWF:Ag, ANG-2, and P-selectin) from BOECs (Fig. 3). In contrast, we observed that BOECs stimulated with HR histone fraction and UH had less VWF:Ag in the cell medium than the untreated control. We attribute this result to the ability of HR histone fraction and UH to bind and sequester VWF to the culture plate, collagen, or endothelial surface, which may interfere with its detection. UH has been shown to bind to the A1 domain of VWF [18], which we confirmed in our soluble binding assay (Fig. S2).

Previous studies have demonstrated that histones induce endothelial cell apoptosis [22,34]. Interestingly, we demonstrated that WPB release by histones was attenuated by calcium chelation and by preincubation of endothelial cells with a pan-caspase inhibitor, suggesting

that histone-induced WPB exocytosis and apoptosis are interrelated processes. Additionally, Chabaan *et al.* have shown that negatively charged molecules neutralize histone cytotoxic activities [39]. Here, we demonstrated that altering cell surface charge by using a highly positively charged polymer (protamine sulfate) or by blocking phosphatidylserine (annexin V) reduced histone-mediated WPB release.

Collectively, our *in vivo* and *in vitro* studies suggest a role for histones in regulating platelet activation and VWF secretion from endothelial cells. Upon WPB secretion, ultralarge VWF multimers remain anchored to the endothelial surface, and bind platelets under shear [40]. We demonstrated that histone-stimulated BOECs had a greater capacity to form VWF–platelet ‘strings’ than histamine/PMA-treated or untreated BOECs (Fig. 4). The greater response with histone treatment may be related to enhanced endothelial VWF release, with concomitant platelet activation by residual histones potentially retained in the flow chamber.

Systemic histone release has been shown to contribute to microvascular thrombosis, as microthrombi have been observed in lung and kidney sections of histone-treated mice [34,39], similarly to what is observed in inflammatory pathologies complicated by disseminated intravascular coagulation. Although histones induce a procoagulant state through platelet activation, thrombin generation, and WPB exocytosis, histone infusion in mice has been shown

Fig. 7. Plasma cell-free DNA (CF-DNA) and Weibel–Palade body contents are increased but not correlated in acute inflammation. von Willebrand factor antigen (VWF:Ag), angiopoietin-2 (ANG-2) and CF-DNA levels in patients with sepsis ($n = 50$) as compared with old controls ($n = 67$) (A). VWF:Ag and ANG-2 levels were not correlated with CF-DNA levels (VWF:Ag, $-r = 0.24$, $P = 0.10$; ANG-2, $-r = 0.19$, $P = 0.21$) (B). VWF:Ag (C) and CF-DNA (D) levels in a murine model of lipopolysaccharide (LPS)-induced endotoxemia ($n \geq 6$). Data are shown as mean values $\pm$ standard error. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. C, control; S, sepsis.

to mediate a bleeding phenotype in a tail-bleed model [20]. Using an intravital model of arterial thrombosis, we demonstrated that histones prevented the formation of a stable occlusion as compared with saline-treated controls, which we hypothesized was attributable to thrombocytopenia. We confirmed that reconstitution with normal, murine platelets could restore localized thrombosis, highlighting the importance of platelets in arterial thrombus formation and the severity of the coagulopathy associated with systemic histone toxicity (Fig. 2).

As DAMP-induced WPB exocytosis may occur in thromboinflammatory pathologies, we assessed the association between DAMPs and WPB under conditions of chronic (aging) and acute (sepsis) inflammation. For these studies, we measured plasma levels of the DAMP, CF-DNA, which is released from damaged cells along with histones, and is frequently employed as a surrogate marker for plasma chromatin release during apoptosis, necrosis, or NET formation [29,41].

Aging is characterized by low-grade chronic inflammation derived from accumulation and impaired resolution of innate immune challenges, leading to prolonged elevations in the levels of inflammatory biomarkers [42,43], including CF-DNA [44], and accumulation of prothrombotic mediators, including an increase in the plasma VWF level [28]. This predisposes the aging population to thrombotic events, including myocardial infarction [17] stroke [45], and deep vein thrombosis [46]. We demonstrated that WPB contents and CF-DNA levels increased in a coordinated fashion in both old humans and old mice (Fig. 6). The correlation between plasma WPB contents and CF-DNA levels may be driven by DAMP-mediated endothelial activation and/or by common inflammatory mediators that induce their concomitant release.

Sepsis is an acute reaction to a systemic pathogenic microorganism insult, resulting in uncontrolled activation of inflammation, with concomitant activation of the

coagulation system, resulting in pathologic immunothrombosis [47]. Previous reports have demonstrated that VWF [48], CF-DNA [29] and nucleosome [9] levels are elevated in patients with severe sepsis and in human LPS trials [49,50]. We further demonstrated that, in both septic patients and a murine model of endotoxemia, plasma levels of WPB-released proteins and CF-DNA were elevated; however, no correlation was observed (Fig. 7). One possible explanation for this is that DAMPs are markers of cell death, and can be released from apoptotic, necrotic and NETting cells. Conversely, WPBs are formed only by endothelial cells, and release their contents on demand and in limited quantities. Owing to the complexity of the proinflammatory response in sepsis, it is likely that, although DAMPs may contribute to some of the WPB release in this context, other proinflammatory mediators may also play a significant role.

Collectively, these studies suggest that DAMPs induce endothelial apoptosis, and may contribute to the procoagulant state observed in inflammatory pathologies by facilitating the release of WPBs. These studies suggest that DAMP-mediated coagulation activation may serve as a possible novel therapeutic target for prophylactic anticoagulation in pathologic immunothrombosis.

Addendum

A. Michels, S. Albáñez, L. L. Swystun, and D. Lillicrap designed experiments and interpreted data. A. Michels, S. Albáñez, J. Mewburn, and K. Nesbitt performed experiments. A. Michels and L. L. Swystun wrote the manuscript. S. Albáñez, L. L. Swystun, T. J. Gould, P. C. Liaw, P. D. James, and D. Lillicrap edited the paper.

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Disclosure of Conflict of Interests

P. James receives research funding from Bayer, CSL Behring, and Octapharma, and honoraria from Baxalta, Biogen, Octapharma, and CSL Behring, and participates on advisory boards for CSL Behring, Baxalta, and Biogen. D. Lillicrap receives research funding from Biogen, Bayer, CSL-Behring, and Octapharma, and participates on advisory boards for Baxalta, CSL-Behring, and

Biogen. The other authors state that they have no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. VWF deficiency has no effect on thrombocytopenia following histone infusion.

Fig. S2. VWF–histone binding interferes with VWF detection *in vitro*.

Fig. S3. Blood outgrowth endothelial cells do not stain positively for TLR-2 or TLR-4 by flow cytometry.

Fig. S4. Lysine-rich histones stimulate Weibel–Palade body release from HUVECs by a caspase-dependent, calcium-dependent and charge-dependent mechanism.

Fig. S5. VWF and DAMP concentrations are not correlated in the absence of chronic inflammation.

Fig S6. Plasma VWF levels do not modify the release of DAMPs.

Table S1. Alignment search summary for human versus bovine histone subsets.

Table S2. Summary of septic patient inclusion data.

Video S1. (a,b) Histones mediate VWF–platelet string formation.

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