**The Role of Extracellular Histones in Haematological Disorders**

**Short Title: Extracellular Histones in Haematological Disorders**

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**Summary**

Over the past decades, chromosomal alterations have been extensively investigated for their pathophysiological relevance in haematological malignancies. In particular, epigenetic modifications of intra-nuclear histones are now known as key regulators of healthy cell cycles that have also evolved into novel therapeutic targets for certain blood cancers. Thus, for most haematologists, histones are DNA-chained proteins that are buried deep within chromatin. However, the plot has deepened with recent revelations on the function of histones when unchained and released extracellularly upon cell death or from activated neutrophils as part of neutrophil extracellular traps (NETs). Extracellular histones and NETs are increasingly recognized for profound cytotoxicity and pro-coagulant effects. This article highlights the importance of recognizing this new paradigm of extracellular histones as a key player in host defence through its damage-associated molecular patterns, which could translate into novel diagnostic and therapeutic biomarkers in various haematological and critical disorders.

**Keywords:** Extracellular histones, nucleosomes, neutrophil extracellular traps (NETS), cell death, thrombin.

**Background**

To most haematologists, the word “histones” conjures associations with epigenetic regulation of chromatin because of the well-recognized role of post-translational modifications of histones in the pathogenesis of haematological malignancies like leukaemia, lymphoma, multiple myeloma and myelodysplastic syndrome (Bhalla and List 2004, Fong*, et al* 2014). However, there has been increasing evidence of the unexpected role of histones as arbiters of cell death and tissue damage when outside their natural environment. This article brings this new consideration into focus to better harmonize understanding of histones in haematology.

**Extracellular histones**

Histones are 5 cationic proteins (H1, H2A, H2B, H3 and H4) in the nucleus of eukaryotic cells. H2A, H2B, H3 and H4 are core histones, which arrange into an octamer from two copies of each, around which 146-147 DNA base pairs wrap tightly to chain histones into position. This histone-DNA complex constitutes the “nucleosome” as the basic building block of chromatin to which linker histone (H1) binds. This compact structural configuration enables genetic regulation (Cheung and Lau 2005) but upon cell death, unwind into its components. Extracellular histones are not usually detectable in the circulation due to rapid clearance (Gauthier*, et al* 1996) but when cell death is extensive, high levels appear and convey cytotoxic effects (Xu*, et al* 2009). In patients, high circulating levels of nucleosomes and histones are present in autoimmune disorders (Chen*, et al* 2014b), sepsis (Alhamdi*, et al* 2015a, Ekaney*, et al* 2014) and trauma (Abrams*, et al* 2013b). There is now strong evidence that such high levels not only indicate cell death but contribute to the pathogenesis of sepsis (Alhamdi*, et al* 2015a, Xu*, et al* 2009), trauma (Abrams*, et al* 2013b) and pancreatitis (Ou*, et al* 2015). While the cytotoxic and pro-coagulant roles of circulating histones and cell-free DNA are increasingly well-recognized, there remains controversy over whether all forms of released nucleosomes express pathogenic effects. It appears that when nucleosomes are released in intact form, DNA-chained histones are not cytotoxic (Abrams*, et al* 2013b). However, when nucleosomes degrade into free histones, DNA or fragments of histone-DNA complexes, exposed histones can mediate pathogenic effects. Indeed, incubating histones with DNA (to form histone-DNA complexes that are not in compact nuclear configuration) did not inhibit the ability of histones to potentiate thrombin generation (Ammollo*, et al* 2011). It remains to be deciphered which forms of nucleosomes or their individual components are predominant in the circulation during systemic inflammation and extensive cell death.

Cell death is not the only mechanism of histone and DNA release. This can also occur following activation from nucleated cells, especially from neutrophils as neutrophil extracellular traps (NETs), which is an amalgam of intracellular and nuclear material including microbicidal enzymes, e.g. myeloperoxidase and elastase (Brinkmann*, et al* 2004). This process known as “NETosis” is a defence mechanism aimed at trapping and killing pathogens (Branzk*, et al* 2014, Brinkmann*, et al* 2004). However, exposed histones within NETs can cause collateral cell damage with involvement in arterial (Massberg*, et al* 2010), venous (Brill*, et al* 2012) and microvascular (Abrams*, et al* 2013b) thrombosis. DNase treatment can breakdown NETs in vivo and remove DNA but only partially attenuate tissue injury because histones are not removed by DNase (Kolaczkowska*, et al* 2015). This emphasizes the important and predominant cytotoxic role of histones and their involvement in activating several pathways e.g NFκB (Allam*, et al* 2012), mitogen-activated protein kinases (Huang*, et al* 2011), NLRP3 inflammasome (Huang*, et al* 2013) and toll-like receptor (TLR) (Allam*, et al* 2012, Huang*, et al* 2013, Xu*, et al* 2011) with inflammatory and detrimental consequences.

**Extracellular histones in blood**

*Specific effects on endothelial cells*

As the active interface between blood and tissues, the vascular endothelium plays a crucial role in maintaining homeostasis and vascular patency. Endothelial cells form the lining of this vast circulatory system; estimated at 3000-6000 m2 (Van der Linden*, et al* 2012) which is equivalent to the size of a football pitch. Indeed, the vascular endothelium can be considered as the largest organ (Endemann and Schiffrin 2004) and its dysfunction has major implications clinically, especially in potentiating multiple organ failure (MOF). In this context, the contribution of extracellular histones to endothelial dysfunction has been extensively studied (Abrams*, et al* 2013b, Ekaney*, et al* 2014, Xu*, et al* 2009). Histones can bind endothelial cell membranes and increase permeability (Abrams*, et al* 2013b). Of translational relevance is the strong correlation between circulating histone levels in trauma patients and soluble thrombomodulin (sTM), a marker of endothelial injury (*rs*=0.55, *P*<0.0001) (Abrams*, et al* 2013b). Also, when such patients’ plasma are incubated with cultured endothelial cells, significant cell death is induced at histone concentrations ≥50 µg/ml (Abrams*, et al* 2013b).

As endothelial cells are integral to haemostatic control, perturbation of pro- and anti-coagulant pathways are affected by histones. Histones enhance tissue factor (TF) expression by endothelial cells in a dose- and time-dependent manner without affecting TF pathway inhibitor (TFPI) expression (Yang*, et al* 2015). This is dependent on TLR-4, TLR-2 receptors and requires NFκB inflammatory activation (Yang*, et al* 2015). However in patients with trauma, a correlation between high circulating histones and TFPI levels was demonstrated along with a significant association between histones, activated protein C (APC) and tissue type-plasminogen activator (tPA) (Kutcher*, et al* 2012). Although these clinical observations do not establish cause-effect relationships, they suggest that high circulating histones might play a role in activating or releasing anti-coagulant and fibrinolytic molecules from endothelial cells. Indeed, histones can enhance release of ultra-large multimers of von Willebrand factor (vWF) (Lam*, et al* 2015), which is supported by observations of histone infusion into mice increasing circulating vWF levels (Brill*, et al* 2012).

In addition, pathogen-derived histones, e.g. from *Plasmodium falciparum* induce endothelial barrier dysfunction and the release of interleukin-8 (IL-8) by endothelial cells (Gillrie, et al 2012). This highlights that histone-mediated pro-inflammatory and endothelial barrier disruption are linked to the conserved nature of histone structure across different species. These findings suggest a multi-factorial role for histones on endothelial cells.

*Specific effects on haematopoietic cells*

Histone infusion into mice causes rapid and profound thrombocytopenia (Fuchs*, et al* 2011), which is mediated by histone-induced platelet aggregation. The process requires both histone-induced calcium influx to activate αIIbβ3 integrins and crosslinking of histone-coated platelets and αIIbβ3 by fibrinogen to form micro- and macro-platelet aggregates, respectively, with H4 and H3 being the most potent (Fuchs*, et al* 2011). Clinically, histone-associated thrombocytopaenia (HaT) has been recently demonstrated with high histone levels on intensive care unit (ICU) admission strongly predicting moderate-severe thrombocytopenia (platelets <100x109/L) (AUC=0.893) (Alhamdi*, et al* 2016). These clinical findings, together with observations in animal studies (Abrams*, et al* 2013a, Fuchs*, et al* 2011),suggest that histones are major mediators of thrombocytopaenia in vivo.

HaT would likely be associated with a pro-thrombotic tendency as H3 and H4 induce exposure of phosphatidylserine (PS) and expression of factor V (FV) and P-selectin (Semeraro*, et al* 2011) to promote thrombin generation. Polyphosphate is also released from platelets, which can promote thrombin generation by accelerating FV and factor XI activation, while also acting as an important mediator between coagulation and inflammation (Travers*, et al* 2015, Zhu*, et al* 2015). These data suggest that histones increase platelet pro-coagulant activity by multiple mechanisms.

As for red blood cells (RBCs), histones also induce PS exposure (Semeraro*, et al* 2014), which is primarily mediated by H4-induced calcium influx to increase prothrombinase activity (Semeraro*, et al* 2014). However, it appears that histones preferentially bind platelets rather than RBCs in vivo to induce profound thrombocytopaenia without detectable effects on RBC count (Fuchs*, et al* 2011, Nakahara*, et al* 2013). As such, histones might not play such a major role in haemolysis as compared to thrombocytopaenia but this requires further studies.

As to white blood cells (WBCs), histones have a bi-directional relationship. At one end, histones stimulate neutrophils to release NETs (Abrams*, et al* 2013b) but NETs also induce histone release (Martinod and Wagner 2014). Histones stimulate release of pro-inflammatory cytokines from WBCs, including IL-6 and TNFα (Abrams*, et al* 2013b, Xu*, et al* 2011), thereby exacerbating inflammation. NETs can also prime macrophages to release cytokines (Warnatsch*, et al* 2015). At a separate level of regulatory control, histone-induced IL-6 would further induce C reactive protein (CRP) (Ganapathi*, et al* 1991), which could bind and neutralize histone cytotoxicity (Abrams*, et al* 2013a) (Figure 1). This suggests a conserved mechanism that would regulate host responses to injury.

*Specific effects on coagulation*

Thrombin generation is a pivotal event that can manifest in diverse and overlapping functions through pro- and anti-coagulant as well as pro- and anti-fibrinolytic consequences (Toh and Alhamdi 2013). When histones are infused in vivo, they induce an increase in thrombin-anti-thrombin (TAT) levels, which can be abrogated by anti-histone antibodies (Abrams*, et al* 2013b). As highlighted above through interactions with endothelial and haematopoietic cells, histones can significantly promote coagulation activation and thrombin generation. However, H4 has been described to bind prothrombin causing auto-activation, which implies direct thrombin generation that is independent of TF or factor XII activation (Barranco-Medina*, et al* 2013). Whether H4-induced prothrombin auto-activation is physiologically relevant remains unclear, especially as the effect takes approximately 8 hours (Barranco-Medina*, et al* 2013). Furthermore, H4-induced prothrombin auto-activation to thrombin could be held-in-check by a negative feedback mechanism as the generated thrombin can gradually degrade H4 (Barranco-Medina*, et al* 2013). This could be a protective mechanism that prevents dissemination of direct histone-induced thrombin generation but requires further investigation.

Histone-induced auto-activation mechanism does not appear to be confined to prothrombin as histone binding to pro-plasma hyaluronan-binding protein (pro-PHBP) (factor VII activating protease) also results in auto-activation (Yamamichi*, et al* 2011). This would suggest that circulating histones might activate the extrinsic pathway independent of TF activity. However, the physiological consequences of this histone-induced pro-PHBP auto-activation remains unclear, especially as enzymatic activity of the active protease has not been demonstrated (Yamamichi*, et al* 2011) amid reports that FVII is particularly resistant to activation by PHBP (Stavenuiter*, et al* 2012).

The intricate and fine balance between thrombin and APC (Dutt and Toh 2008) can also be unhinged by histones. Histones suppress thrombomodulin (TM)-dependent protein C activation (Ammollo*, et al* 2011) and this may reflect another homeostatic regulatory relationship to the ability of APC to proteolytically inactivate circulating histones (Xu, et al 2009) (Figure 1). Therefore, delivering appropriate APC levels in vivo might be relevant in recalibrating haemostatic dysfunction in critical illness and revive clinical interest for therapeutic enhancement of APC activity. Indeed, TM injection into mice significantly abrogates histone-induced cytotoxicity and procoagulant effects by binding histones (Nakahara*, et al* 2013).

As for the fibrinolytic system, histones, DNA and NETs increase clot stability through increased resistance to fibrinolysis (Longstaff*, et al* 2013). Histones and DNA alter fibrin architecture in plasma clots and DNase treatment can enhance clot lysis to suggest that NETs are major contributors to reduced lysis susceptibility (Varju*, et al* 2015). Table I presents a summary of proposed mechanisms underlying histone-mediated cytotoxicity, procoagulant and inflammatory effects.

**Relevance of extracellular histones in haematological disorders**

*Role in deep venous thrombosis (DVT)*

Nucleosomes and NETs have been implicated in DVT pathogenesis (Brill*, et al* 2012, van Montfoort*, et al* 2013). In a mouse DVT model induced by inferior vena cava (IVC) stenosis, high DNA levels were detectable within 6 hours with immunohistochemical examination of thrombi showing citrullinated H3, a marker of NETs, in close proximity to vWF (Brill*, et al* 2012). This, together with the observation that intravenous histone infusion into mice increases vWF levels and promotes DVT after IVC stenosis, indicate a major role for nuclear breakdown products in DVT development (Brill*, et al* 2012). The clinical relevance of these observations was illustrated in a case-control study of 150 patients with symptomatic DVT and 195 DVT-free patients (controls), which showed a significant dose-dependent associations between circulating nucleosome levels and DVT development (van Montfoort*, et al* 2013).

*Role in sickle cell disease (SCD)*

The potential contribution of histones and nucleosomes to SCD pathogenesis is suggested by an observational clinical study demonstrating significantly elevated levels of nucleosomes and elastase-α1-antitrypsin complexes, a marker of neutrophil activation, in 74 patients with steady state SCD compared to 24 ethnically-matched controls (Schimmel*, et al* 2013). In 70 patients who experienced a painful crisis, levels of both biomarkers were significantly elevated compared to steady state and the two markers correlated significantly with each other (Schimmel*, et al* 2013). Although these observations suggest a potential role for nucleosomes and NETs in SCD, such elevated levels may indicate increased cell death in SCD patients rather than a causative relationship. However, haem from haemolysed RBCs can directly stimulate NETosis (Chen*, et al* 2014a). Using a humanized mouse SCD model, NETs in lungs and elevated NETs components in plasma were associated with increased mortality (Chen*, et al* 2014a). Using either DNase treatment or reducing plasma haem concentrations in this SCD model, reduced NETs significantly (Chen*, et al* 2014a).

*Role in disseminated intravascular coagulation (DIC)*

DIC reflects the consequences of increased thrombin generation in vivo. In most instances, there is loss of the exquisitely regulated process of clot formation with uncontrolled systemic coagulation activation leading to an increased risk of death (Toh and Alhamdi 2013). The combined pro-coagulant effects of circulating histones on endothelial cells, platelets, coagulant factors and anti-coagulant pathways may be pivotal to DIC pathogenesis. Histone infusion into mice results in increased TAT levels, excessive intravascular thrombosis and thrombocytopenia, which are key features of DIC (Abrams*, et al* 2013b, Fuchs*, et al* 2011, Nakahara*, et al* 2013). Importantly, circulating levels of nucleosomes and cell-free DNA correlate with severity of coagulopathy in 199 patients with suspected DIC (Kim*, et al* 2015). Circulating levels of nucleosomes and cell-free DNA predict DIC with area under receiver operating characteristics curve of 0.700 and 0.728, respectively (Kim*, et al* 2015). As the study was limited to 20 healthy controls and did not include matched critically ill patients without DIC (Kim*, et al* 2015), further confirmation is required. Therapeutically, the neutralization of histone cytotoxicity by sTM (Nakahara*, et al* 2013) may be one mechanism underlying the promise of sTM therapy in patients with DIC (Vincent*, et al* 2013).

**Relevance of extracellular histones in critical illness**

The description of damage associated molecular pattern molecules (DAMPs) refers to proteins that change from physiological intracellular functions into pathological extracellular effects when released following cell injury. Prime examples includ**e** high mobility group box-1 (HMGB1) (Scaffidi*, et al* 2002) and histones(Abrams*, et al* 2013b, Alhamdi*, et al* 2015a, Alhamdi*, et al* 2015b, Huang*, et al* 2011, Xu*, et al* 2011, Xu*, et al* 2009). Histone-induced damage to the endothelium increases vascular permeability and there is strong clinical association between circulating histone levels and noradrenaline concentrations administered to achieve haemodynamic stability in septic patients (Alhamdi*, et al* 2015a). Rather distinctively is the ability of extracellular histones to mediate distant organ injury as exemplified in two separate in vivo models with accompanying clinical studies.

Firstly, a peritonitis model showed histone-dependent distant injury to the heart causing mechanical and electrophysiological dysfunction that was abrogated by anti-histone antibody therapy (Alhamdi*, et al* 2015a). Of translational relevance is that in patients with severe sepsis without a history of cardiac disease, circulating histones strongly correlated with new-onset left ventricular dysfunction and arrhythmias (Alhamdi*, et al* 2015a). Furthermore, circulating histones are a new and common cause for elevated cardiac troponin (cTn) levels as histone infusion into mice induces time-dependent cTn elevation, which could be abrogated using anti-histone antibodies (Alhamdi*, et al* 2015b). There is also a strong correlation between circulating histone and cTn levels during sepsis in both animal models (r=0.877, *P*<0.001) and patients (r=0.650, *P*<0.001) (Alhamdi*, et al* 2015a).

Secondly, a severe non-thoracic trauma model showed that acute lung injury (ALI) could be abrogated by anti-histone antibody therapy (Abrams*, et al* 2013b). Equally, in 52 patients with severe trauma without thoracic involvement, there was significant correlation between circulating histone levels with ALI (Abrams*, et al* 2013b). As for the mechanism of histone release, studies using mouse models of ALI and sepsis revealed the requirement of complement receptors C5aR and C5L2 as well as NLRP3 inflammasome (Bosmann*, et al* 2013, Kalbitz*, et al* 2015).

Furthermore, NETs form in the pulmonary microvasculature of mice and patients with transfusion-associated lung injury (TRALI) (Caudrillier*, et al* 2012, Thomas*, et al* 2012). This was associated with increased circulating NETs components (Caudrillier*, et al* 2012, Thomas*, et al* 2012), which could be abrogated with anti-histone antibody or DNase treatment to confer protection against TRALI (Caudrillier*, et al* 2012, Thomas*, et al* 2012).

**Conclusion**

The release of intra-nuclear material into the circulation upon extensive cell injury presents DAMPs with cytotoxic effects that could shift from adaptive and protective functions to becoming maladaptive with adverse clinical consequences. Resulting from disintegration of nucleosomes and separation from DNA, histones reverse function when outside its normal intra-nuclear environment; i.e. from helping cell regulation to causing cell death. Although this is important as part of host defence against invading pathogens, there is the potential for collateral damage to tissues, MOF and death. With an increasing number of animal modelling studies and clinical cohorts highlighting this pathogenic relevance, circulating histone levels could be relevant biomarkers in prognostication and monitoring for adverse outcomes. Its proximate role in the cross-talk between coagulation, inflammation and innate immunity (Figure 2) makes it a potentially useful target in haematological disorders of the circulation and critical illness, especially with promising protective effects using anti-histone antibodies (Abrams*, et al* 2013b, Alhamdi*, et al* 2015a, Alhamdi*, et al* 2015b, Xu*, et al* 2009), APC (Xu*, et al* 2009), recombinant TM (Nakahara*, et al* 2013) and heparin (Fuchs*, et al* 2011, Wildhagen*, et al* 2014).

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**Figure legends**

**Figure 1. Major histone-induced effects and regulatory consequences.**

Extracellular histones generated as a consequence of endothelial cell damage promote direct and indirect (via platelets) thrombin (IIa) generation, which in turn facilitates the generation of activated protein C (APC) to proteolytically inactivate histone cytotoxicity. Histones are also neutralised by C reactive protein (CRP), which is induced by interleukin 6 (IL6) that is released from white blood cells (WBCs) stimulated by histones.

**Figure 2. Histones are at the centre of interactions between coagulation, innate immunity and inflammatory pathways.**

Histones induce a pro-coagulant phenotype by mechanisms that include suppressing thrombomodulin (TM)-dependent generation of activated protein C (APC), inducing release of platelet polyphosphate and increasing thrombin generation. Histones are also part of innate immunity by inducing release of neutrophil extracellular traps (NETs) to immobilize and kill pathogens through its microbicidal effects. Release of histones is also dependent on the C5a complement receptor. The pro-inflammatory effects of histones involve activating inflammasomes, releasing pro-inflammatory cytokines and activating the NFκB pathway.