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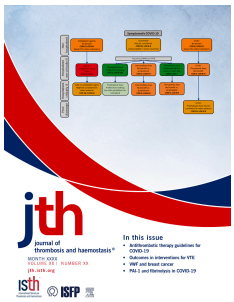

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Cell-free histones and the cell-based model of coagulation

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Abstract

The cell-based model of coagulation remains the basis of our current understanding in clinical haemostasis and thrombosis. Its advancement on the coagulation cascade model has enabled new pro-haemostatic and anticoagulant treatments to be developed. In the past decade, there has been increasing evidence of the procoagulant properties of extracellular, cell-free histones (CFHs). Although high levels of circulating CFH released following extensive cell death in acute critical illnesses, e.g. sepsis and trauma, have been associated with adverse coagulation outcomes including disseminated intravascular coagulation, new information has also emerged on how its local effects contribute to physiological clot formation. CFHs initiate coagulation by tissue factor (TF) exposure, either by destruction of the endovascular barrier or induction of endoluminal TF expression on endothelia and monocytes. CFH can also bind prothrombin directly, generating thrombin via the alternative prothrombinase pathway. In amplifying and augmenting the procoagulant signal, CFHs activate and aggregate platelets, increases procoagulant material bioavailability through platelet degranulation and Weibel-Palade body exocytosis, activates intrinsic coagulation via platelet polyphosphate release, and induces phosphatidylserine exposure. CFHs also inhibit protein C activation and downregulate thrombomodulin expression to reduce anti-inflammatory and anticoagulant effects. In consolidating clot formation, CFHs augment the fibrin polymer to confer fibrinolytic resistance and integrate neutrophil extracellular traps into the clot structure. Such new information holds the promise of new therapeutic developments, including improved targeting of immunothrombotic pathologies in acute critical illnesses.

Keywords: Extracellular histones, thrombin, coagulation, thrombosis, and neutrophil extracellular traps (NETs).

Introduction

A little more than two decades has passed since the cell-based model of haemostasis was conceptualised by Maureane Hoffman and her colleagues [1]. By incorporating the cellular contribution to clot generation and regulation, the cell-based model advanced its predecessor - the traditional coagulation cascade model - by better reflecting in vivo haemostasis. Haemostatic physiology was illustrated through three distinct yet overlapping stages: initiation, amplification, and propagation [1]. Despite its development at the turn of the millennium, the cell-based model remains the foundation of current understanding and clinical practice in haemostasis and thrombosis [1]. In view of the significant advances in the field since, which have been catalysed by the COVID-19 pandemic, can this model still apply especially with the increased recognition of the cross-talk between coagulation, inflammation, and innate immune activation?

The term ‘immunothrombosis’ has been used to encapsulate the pathological consequences of such a cross-talk [2]. Proposed nearly a decade ago, immunothrombosis reflects the highly conserved relationship between the immune and haemostatic pathways that stem from common evolutionary roots in the response to injury. These pathways tend to be understood as distinct entities, but such conceptual singularity should no longer hold given the new surge of focus in how immunothrombotic dysfunction is a key contributor to adverse outcome in acute critical illnesses [3]. The urgent need to address the high mortality associated with these varied disorders, which include sepsis and acute pancreatitis, strongly argues for better understanding and teaching of immunohaemostasis and immunothrombosis with translation into better diagnostic and therapeutic tools.

A prominent candidate featured in this conversation is the neutrophil extracellular trap (NET) [4]. Initially studied separately by different disciplines, it is now clear that its role converges pathogen immobilisation and killing with coagulation activation and clot formation [4]. Its interaction with fibrin to reinforce thrombus formation is important and a hallmark of COVID lung at postmortem [5, 6]. Like fibrin, NET formation is an end-product of pathway activation. From a therapeutic perspective, it might be more important to understand upstream effectors of NETosis just as the understanding of coagulation

activation, from the cascade to the cell-based models, have expanded the armoury of therapeutic tools in both bleeding and thrombotic disorders [7-9].

Among the different affectors of NETosis, the cell-free histone (CFH) has emerged as a molecule that could be highly relevant to immunothrombosis [10]. There has been increasing evidence of its diverse actions on many components of the coagulation, inflammation, and innate immune pathways. Understanding this evolutionarily ancient protein, with its different but converging responses to injury, might advance how haemostasis and thrombosis knowledge could better address the many diseases that have immunothrombotic pathogenesis at its core. This review aims to refresh the cell-based model of haemostasis from the perspective of our current knowledge of CFH function and how it could also tip over into thrombosis. Other DAMPs have also been reported to have procoagulant effects. Examples include high mobility group box-1 (HMGB-1), which activates platelets [11], and cell-free DNA, which electrostatically activates the intrinsic coagulation cascade [12]. Procoagulant, non-DAMP intracellular material may also be released alongside DAMPs, e.g. neutrophil elastase during NETosis, which degrades innate anticoagulants including tissue factor pathway inhibitor (TFPI). This breadth of both procoagulant material and mechanisms makes its comprehensive inclusion beyond the scope of this article.

Cell-free histones

Histones are a group of highly conserved eukaryotic proteins, which are well known for their intranuclear function in facilitating chromatin packaging and the regulation of genetic expression through post-translational modifications (PTM) [13]. Thus, the sub-grouping of histones are by intranuclear function: core (H2A, H2B, H3, and H4), and linker (H1 and H5) [13]. Pairs of H3-H4 and H2A-H2B dimers come together as octamers, which forms nucleosomes by wrapping a 146 base pair length of DNA around itself. Through linker histones, sequential nucleosomes form chains into higher order chromatin, which are supercoiled into individual chromosomes.

This level of intranuclear organisation is made possible by the highly cationic or positive charge of histones, conferred by a primary sequence that is rich in either arginine (H3 and H4) or lysine residues

(H1, H2A, and H2B) [14]. This cationicity facilitates histone-DNA association by electrostatically interacting with the highly anionic phosphate backbone of DNA. Simultaneously, this interaction neutralises intra-DNA anionic repulsion to facilitate tight DNA compaction. Charge altering PTM of arginine and lysine residues modifies the degree of anionic charge neutralisation for intra-DNA repulsion that regulates genomic expression by manipulating DNA accessibility [14, 15].

By contrast, the role of extracellular histones is a relatively recent discovery [10]. CFHs gained prominence at the turn of the last decade when Xu et al discovered its presence as a substrate of activated protein C (aPC), which led to demonstration of its significant mediation of death in sepsis models [10]. It is now widely recognized that CFHs serve as a secondary hit in many critical illnesses with extensive cell death, most prominently in those with severe sepsis [10, 16], pancreatitis [17, 18], and trauma [19]. Analyses have demonstrated the potential of CFH as a prognostic biomarker for coagulopathy, organ injury and mortality in these diseases [3, 16, 18-22].

Circulatory release of histones is inevitable upon pathological cell death, or by NETosis, where CFHs are generated following stepwise degradation of liberated chromatin into its fundamental components (figure 1) [23, 24]. Current understanding of the metabolism of extruded nuclear material remains limited [23-25]. From what is known, extracellular chromatin is digested by DNases-1 and -3 to release individual nucleosomes [26, 27]. Nucleosomes have an estimated circulating half-life of 15 minutes and are eliminated by the liver [25, 28, 29]. Persistence of circulating nucleosomes allows for its dissociation into cell-free DNA and histone octamers, whereby histone octamers undergo further stepwise degradation into histone dimers before forming CFHs.

Unfortunately, the very same histone cationicity that is essential for intranuclear biology is responsible for the majority of its extracellular pathogenicity through indiscriminate binding to anionic components of the circulation and vasculature. This facilitates its broad range of biological impact extracellularly, which spans the triumvirate of innate immunity, inflammation, and the coagulation, and is testament to its evolutionary relationship to host responses upon insult and injury. The charged-mediated antimicrobial properties of CFHs are also important. These effects may be NETs-associated, both as a trigger of NETosis and as a functional component of NETs, or independently in circulatory form.

Additionally, CFHs are evolutionarily co-opted as damage associated molecular patterns (DAMPs) and exert its own unique inflammatory signature through Toll-like receptor (TLR) signalling [30, 31]. In terms of classic complement, CFHs act as negative regulatory elements to ameliorate its cytotoxic collateral by inhibiting membrane attack complex formation [32].

The ability of positively charged CFHs to affect negatively charged surfaces, alongside its immunostimulatory effects, are also highly relevant to coagulation. Semeraro et al discovered that CFHs dose-dependently enhanced thrombin generation in citrated, contact factor inhibited, recalcified platelet rich plasma (PRP) [30]. In vivo, histone infusion induced dose-dependent increments in thrombin generation in mice, quantifiable via its surrogate marker thrombin-antithrombin (TAT) complexes [19, 33]. Elevated CFH levels in critically ill patients correlate with circulating TAT levels [16, 19]. Pulmonary and renal microthrombi found following CFHs infusion in animal models bear striking resemblance to those observed in patients with multi-organ failure and critical illnesses, post-mortem [10, 19, 22, 34, 35]. Crucially, the immuno-thrombogenicity of CFHs is dose-dependent and can be ameliorated following anti-histone targeting. This highlights its potential as a therapeutic target, in addition to its prognostic value in a range of life-threatening medical disorders with a shared immunothrombotic pathogenesis [10, 22]. Potential treatment strategies include CFH neutralisers, e.g. aPC [10], desulphated heparin [36] and small polyanions [27], or by its extracorporeal removal [37]. While many of the advancements stemmed from the initial observations of high circulating levels in systemic disorders, the dose-dependency and localised effects of CFHs highlight how injury-released CFH could contribute towards an effective physiological haemostatic response.

Initiation of Coagulation

The cell-based model emphasizes cellular receptors as the primary ignitor of haemostasis, e.g. tissue factor (TF) [38]. TF is constitutively expressed only on extravascular cell surfaces. Thus, haemostatic signals are almost exclusively instigated when an endothelial barrier breach allows its contact and complexing with activated FVII (FVIIa), forming a TFase capable of enzymatic activation of FX [39]. Downstream of this reaction, sufficient activated FX (FXa) is generated to adequately cause a thrombin

spark. Regulation of coagulation initiation relies upon FXa inhibition by TFPI and antithrombin in the fluid phase, thus localising haemostatic activity to TF-bearing cells.

Injury-released CFHs facilitate the site-specific TFase reaction through several ways. One is through the disruption of endothelial barrier integrity to permit circulatory access to the procoagulant sub-endothelium (Figure 1A). CFH cytotoxicity against vascular endothelia is well reported in a range of primary endothelial and established cell lines [10, 19, 33, 40, 41]. The predilection of CFHs for anionic membrane phospholipids [19, 33, 42] compromises endothelial barrier resilience through ionophore-induced apoptosis and gap junction disruption [19, 43].

Without disrupting endothelial integrity, CFHs can also initiate thrombin generation by inducing intravascular TF expression in both endothelia and monocytes (Figure 1B) [40, 44]. Sub-cytotoxic doses of histones can induce endothelial TF expression by 26-fold, with maximal procoagulant activity at 6 hours post-stimulation [40]. Serum from critically ill patients can enhance monocytic TF expression derived from healthy donors ex-vivo and the effect is abrogated in those who have received heparin, a known CFH neutraliser [44]. CFHs induce or increase TF synthesis in both endothelial and monocytic cell lines via TLR2 and partly TLR4 signalling, which in turn mediates upregulation of transcription factors NF-κB and activating protein-1 (AP-1) [40, 44]. The majority of expressed TF is inert and requires an intermediate decryption step to participate in effective extrinsic tenase activity [45]. Whilst the exact mechanism of decryption remains debated [45], the likely candidates, i.e. phosphatidylserine (PS) exposure and protein disulphide isomerase, are induced by CFHs [46].

CFH can induce autoactivation of FVII activating protease (FSAP) [47, 48], which circulates physiologically as a zymogen. When activated, its trypsin-like activity amplifies the procoagulant signal by increasing the circulating volume of FVIIa whilst simultaneously inactivating TFPI [49].

Independent of conventional tenase activity, CFHs can also initiate coagulation by direct common pathway activation (Figure 1C). Histones are known to precipitate prothrombin ex-vivo [50], with the H4 fraction binding prothrombin to enable autoactivation in vitro [51]. Its clinical relevance is uncertain as the experiment was only conducted in buffer, with autoactivation requiring a minimum of 8 hours

and remained incomplete despite prolonged incubation to 84 hours [51, 52]. However, other work has demonstrated that binding of arginine rich histone fractions to prothrombin facilitated its cleavage by FXa to generate thrombin [52]. Conventional prothrombinase activity requires FXa binding to activated FV (FVa) in a calcium and phospholipid dependent fashion. In contrast, CFH-prothrombin binding results in alternative prothrombinase activity, with CFHs acting as an alternative co-factor to FVa [52]. Importantly, thrombin generation by alternative prothrombinase requires significantly less FXa, thus reducing the threshold for thrombin generation [52]. Additionally, the alternative prothrombinase pathway is phospholipid (thus cell activation) independent and contributes to both clot initiation and dissemination of coagulation away from vascular surfaces [52]. Whilst thrombin generation by alternative prothrombinase is less efficient when compared to its conventional counterpart, it remains sufficiently potent in restoring clot generation in haemophilic plasma and mouse models [52].

Amplification of Coagulation

The signal amplitude of thrombin generated during the initiation phase of haemostasis is insufficient for clot formation. Here, the haemostatic contribution of cells - crucially the platelet-endothelial axis - is pivotal in amplifying the thrombin signal towards achieving effective clot formation [53]. Thrombin generated during the initiation phase facilitates localised cellular activation through protease activated receptors (PARs), liberates FVIII from von Willebrand factor (VWF) and proteolytic activates coagulation factors FV, FVIII, FIX, and FXI. Activated platelet and endothelial cells degranulate to contribute substantial procoagulant material, whilst PS expression upon intravascular membrane surfaces facilitates coagulation machinery assembly and proteolytic activity.

CFHs boost coagulation amplification by inducing platelet activation, degranulation and aggregation (Figure 2A). There is increased fibrinogen binding to platelets, VWF release, P-selectin expression, PS expression, and increased neutrophil-platelet aggregation [30, 42, 54, 55] resulting from CFH binding to platelet surfaces and inducing calcium influx [42]. Signal cascades activated include ERK, PI3K-AKT, p38, and NF-κB, which are both TLR (2 and 4) dependent and independent [55], alluding to the existence of other yet unelucidated CFH signal transduction pathways. Platelet degranulation contributes to procoagulant material bioavailability, which includes conventional coagulant enzymes

FV, FVIII (alongside VWF), and FIX, but also platelet polyphosphates (polyP) (Figure 2B) [56]. Thrombin generation is demonstrated to be polyP-dependent in histone-treated, FXII-inhibited platelet rich plasma (PRP) by its abrogation following polyP digestion by alkaline phosphatase [30]. Although polyP is a known contact factor pathway activator, its haemostatic effect is independent of FXII in the presence of histones [30, 57]. PolyP may plausibly exert this effect by accelerating FV activation or via inhibition of TFPI [57, 58].

The VWF-platelet axis is key towards effective clot generation, not merely via its ability to bridge adjacent platelets through glycoprotein IIb/IIIa, but also through VWF recruitment of platelets to areas of endothelial activation [59, 60]. CFHs have been demonstrated to induce endothelial Weibel Palade (WP) body degranulation to release ultra-large VWF multimers into the circulation (Figure 2C) [60-62]. Ultra-large VWF liberated following WP exocytosis are endothelially anchored and bind platelets following mechano-activation under shear [60, 63]. The mechanics of WP-exocytosis were found to be calcium, caspase, and charge-dependent, but independent of TLR2 and TLR4 [60]. The caspase-dependent mechanism suggests that WP exocytosis is part of the endothelial death process, supported by the abrogation of CFH-induced WP degranulation by pan-caspase inhibitors [60]. It is also plausible for CFH-induced, ionophore-mediated calcium influx to trigger WP exocytosis (Figure 3). In vivo, exogenous histones infused into C57BL/6 mice resulted in significantly elevated VWF levels, significant thrombocytopenia, and elevated thrombin-antithrombin complexes when compared to negative controls [61]. However, no significant increases in ultra-large VWF levels were noted. This is attributed to the presence of a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13 (ADAMTS13) in vivo, which is absent in in vitro experiments [61]. However, ADAMTS13 may be proteolyzed by thrombin during the acute phase response, an action postulated to augment local thrombogenic potential by preservation of ultra-large VWF [64].

Both CFH-induced platelet activation and CFH enhancement of platelet-VWF interactions likely contribute towards thrombocytopenia in critical illness [42, 54]. Sublethal infusion of histones depletes ~90% platelets in mice [42, 65], whilst platelet aggregates are major constituents of histone-induced

thrombus in vitro and in vivo [30, 34]. Interestingly, pre-depletion of platelets in mice with anti-GP-1b antibodies conferred some protection against histone-induced death [42].

Propagation of Coagulation

Negatively charged elements embedded within cell surfaces (e.g. PS) is a critical facilitator and accelerant of thrombin generation through assembly of tenase and conventional prothrombinase complexes via electrostatic interactions with the GLA-domain of vitamin-K dependent coagulation proteins [66]. Induction of PS exposure on cell surfaces is essential for this process and CFHs translocate PS from the inner endothelial membrane leaflet to the external [46] by the modulation of flippase-floppase following CFH-induced calcium influx (Figures 3A and 3B). PS surface expression is a well-established marker of CFH cytotoxicity in both platelets and endothelia [33, 54]. However, PS exposure is not unique to these cells only and there is in vitro evidence that CFH activated erythrocytes play a role in thrombogenesis through PS expression [67]. By flow cytometry, CFHs induce formation of small erythrocyte aggregates whilst simultaneously inducing PS expression detectable by annexin V staining [67, 68]. Under purified conditions, CFHs-activated erythrocytes are able to support and accelerate thrombin generation. Additionally, intravascular CFH induced lysis/pyroptosis, regardless of cell type, to generate PS-rich micro-particles (MPs) with significant procoagulant potential [69, 70]. MPs with TF activity, such as those derived from monocytes or endothelial cells, may also be enhanced by CFH-induced PS expression through TF decryption [44].

Clot stability requires individual fibrin strands to be covalently woven into a supportive meshwork through transglutamination by activated factor XIII (FXIIIa). This cross-linking activity is simultaneously capable of covalently integrating a myriad of circulating constituents, including CFHs, into the fibrin scaffold to enhance bio-physical properties (Figure 3C) [71, 72]. In plasma, CFH-crosslinked fibrin confers resistance to clot lysis by exogenous tissue plasminogen activator (tPA) in a FXIIIa-dependent manner, prolonging clot lysis times by up to 50% [71]. This CFH-conferred fibrinolytic resistance is also demonstrable in viscoelastic studies performed on whole blood [71]. CFHs further increase clot robustness through non-covalent interactions with fibrin polymers, encouraging lateral aggregation of fibrin protofibrils into thicker fibrin fibres [71]. Pathological concentrations of

CFHs have also been demonstrated to competitively inhibit plasmin's fibrinolytic activity [71]. Conversely, CFHs-activated FSAP could activate tPA and urokinase plasminogen activators (uPA) to promote fibrinolysis [73]. There is also evidence that FSAP enhances fibrinolysis by altering the bio-physical properties of the thrombus through modification of its fibrinogen substrate [73]. Overall, the relationship between CFH-induced FSAP autoactivation and its net functional impacts upon the coagulation system remains unclear.

Reinforcement of Coagulation

In addition to the cell-based model description of initiation, amplification, and propagation phases of coagulation, there are other effects operating in parallel that consolidate and potentially extend clot formation (Figure 4). One such effect channelled by CFHs is the recruitment and simulation of neutrophils to extrude NETs within the locality of injury and its release [74, 75]. This signal can be further amplified and propagated through histones embedded within released NETs or CFH released into the circulation following NET degradation. Ex vivo quantification by immunoblotting estimates 70% of NET-bound proteins are CFHs with procoagulant potential [76]. Additionally, NETs also act as a physical scaffold upon which platelets, erythrocytes and procoagulant components juxtapose and aggregate, including VWF [77], fibrinogen [78], and TF [79, 80]. Integration of NETs into fibrin enhances clot robustness by conferring tPA-resistance [77]. Furthermore, an enhanced or sustained effect could be thrombogenic. In vivo, neutrophil congestion with NETs are major constituents of the pulmonary thrombotic burden in histone-infused mice [10, 19, 41] and in balloon catheter-induced DVTs in baboons [77].

The procoagulant effect of CFHs is also enhanced by its suppression of the innate protein C (PC) anticoagulant pathway. This pathway depends on the cell surface receptor thrombomodulin (TM) binding thrombin and redirecting its proteolytic activity towards the activation of PC into aPC[81]. aPC, in the presence of its co-factor protein S, proteolytic degrades FVa and FVIIIa for anticoagulant effect. CFHs disrupt this axis by directly interacting with both TM and the zymogen protein C (PC). Both contain anionic domains that can electrostatically interact with cationic CFHs – chondroitin sulfate on TM following glycation of its extracellular domain, and the GLA domain on PC [81, 82]. In vivo

evidence suggests that this electrostatic interaction is exacerbated by the presence of other circulating cationic molecules, including platelet factor 4 (PF4) [81, 82]. CFHs can dose-dependently reduce endothelial surface TM antigen and activity in vitro by downregulating its synthesis through a reduction in cytosolic TM mRNA, rather than through metalloprotease mediated TM-shedding [46]. The mechanism remains unclear but appears to be independent of TLR2 and TLR4 signalling. This mechanism however does not explain the elevation in soluble TM (sTM) levels in patients with elevated CFHs following severe blunt trauma [19]. Shedding occurs plausibly due to actions of other proteases [83], or as a consequence of endothelial cell damage or death. Further suppression of the TM-aPC axis occurs as a consequence of CFH-PC binding, which inhibits PC activation [81].

The enhanced and extended coupling of procoagulant and proinflammatory effects in thrombogenesis have long been recognised and extensively reviewed [84, 85]. Whilst prominently recognised for its anticoagulant effects, the TM-aPC axis plays a crucial role in inflammatory modulation, which can be both dependent or independent of protease activated receptor-1 (PAR-1) [86, 87]. It is therefore conceivable for excess CFH to provoke thrombosis by simultaneously overwhelming both anticoagulant and anti-inflammatory functions of the TM-aPC axis.

As DAMPs, CFHs also exert direct proinflammatory signals [10, 16, 19, 22]. Pathways that have previously been implicated in the inflammation-thrombosis axis can be affected by CFH [88, 89]. For example, CFH interacts with TLRs 2 and 4 to activate MyD88-dependent nuclear signalling and with TLR9 to activate the NLRP3 inflammasome [40, 90, 91]. Limited in vitro data published recently demonstrates that CFH induces a broadside of NLRP3 inflammasome-dependent inflammatory phenotypic changes in primary human endothelial cells [92]. These include dysregulating vascular homeostasis by downregulating nitric oxide synthesis and modifying the endothelial prostanoid profile [92]. There is also enrichment of the local inflammatory cellular milieu by upregulating adhesion receptors (VCAM-1, ICAM-1, P-selectin, E-selectin) and chemokine synthesis [88, 92] and the exertion of proinflammatory, prothrombotic auto- and paracrine effects by the generated cytokine signature (e.g. TNF-α, IL-1β, and IL-6) and reactive oxidative species [91, 92]. This data did not directly demonstrate clot formation. However, when reconciled with pre-existing mechanistic evidence of the inflammation-

thrombosis axis, it supports the notion that CFHs exert a parallel, indirect procoagulant signal via its inflammatory DAMP signature, which could tip physiological immunohaemostatic responses into immunothrombotic pathologies.

Beyond the localised synergization of procoagulant and proinflammatory effects to promote site-specific thrombosis, excess CFH in the circulation can cause generalised hypercytokinaemia. In tandem with an increase in procoagulants (e.g., FVIII and VWF) with corresponding reduction in anticoagulants (e.g. protein S), there is considerable evidence from in vivo and clinical data that very high or sustained levels of CFH can lead to disseminated intravascular coagulation (DIC) [93, 94]. The details are beyond the remit of this review article but notable interactions, which may affect the size and extent of thrombosis includes the ability of some plasma constituents to neutralise CFH toxicity. Several of these are upregulated during the acute phase, including CRP and other pentraxins [33, 95], glycosaminoglycans [35, 96] and soluble TM [34]. While this might suggest an innate regulatory mechanism against CFH toxicity during the acute phase of illnesses, it remains unclear how this balances out dynamically and temporally against histone-quenching plasma constituents that are downregulated during the acute phase, such as albumin and aPC [54].

Discussion

In this review, we utilized the cell-based model to frame how CFHs contribute towards effective haemostasis. The cell-based model emphasizes how coagulation is regulated by properties of cell surfaces in the three overlapping stages of initiation, amplification, and propagation. Such a representation evolved from the paradigm of considering coagulation as a cascading series of enzymatic events. In layering on the effects of CFH on coagulation, this is not cascading in moving from one function to the next but is overlapping, often acting simultaneously and in synergy with other pathways to fortify the host response to injury and potential microbial entry [3, 10, 16, 19, 31, 97]. In keeping with some features of the traditional coagulation cascade model, CFH can act as a novel co-factor, e.g. in the prothrombinase complex, by replacing FVa and the need for phospholipid surfaces to catalyze thrombin generation. Within the remit of the cell-based model, CFHs manipulate cellular elements, e.g. platelets and endothelia, to enhance provision of procoagulant surfaces for enzyme-substrate complex

assembly. Distinct from both theories is how CFHs contribute to haemostatic clot formation by integrating innate immune activation, e.g. through NETS. Histones stimulate NETosis, which acts as a physical and biochemical scaffold for clot formation. This includes NETs-tethered CFHs also exerting its own multitude of procoagulant effects.

From the physiology of haemostasis to the pathology of thrombosis, these described effects of CFH could also be applied to the time-honoured consideration of Virchow's triad, i.e. endothelial injury, hypercoagulability and vascular stasis. All three effects have been detailed above with stasis influenced considerably by the potentiation of NETs. The dose-dependent characteristics of CFH in each of its multiple procoagulant effects suggests that its concentration at local and systemic level plays an important role towards thrombogenesis, coagulation consumption, and the risk of progression towards DIC.

While many of the described mechanisms of CFH in the literature are from in vitro experiments, this review has emphasized when in vivo modelling and ex vivo experiments using patient samples have validated the findings. This is important because the effects of CFH may be affected by its circulatory form and by its complexation or neutralisation by regulatory proteins in the local environment and in circulation. Circulating histones are likely to be a mixture that includes CFHs and DNA-bound histones, which range from nucleosomes to chromatin and NETs. Circulating nucleosome levels have been associated with thrombogenic potential [98, 99], it is however unclear if this is directly attributable to nucleosomes themselves, or from CFHs following nucleosome breakdown. Some in vitro evidence suggests that complete dissociation of histones from DNA is essential for any charge-dependent procoagulant effects to occur [12]. Likewise, this may apply to smaller histone-DNA fragments, which have also been described as potentially thrombogenic. However, the necessity of histone-DNA dissociation for its non-charge dependent procoagulant effects is unclear. For example, incorporation of both DNA and histones into a fibrin clot in vitro confers better fibrinolytic resistance than the individual components alone [71]. CFHs, DNA-histone complexes, and nucleosomes also possess distinct immunostimulatory signatures – mediated by distinct inflammatory signalling pathways, some with synergistic overlap [23]. More importantly, DNA-bound histones lack the electrostatic cytotoxicity

of its unbound form [19]. How the local versus circulating histone composition tips the haemostasis-thrombosis axis remains unclear.

There is also evidence of an intrinsic, integrated regulatory mechanism within the CFHs-coagulation axis, as exemplified by the generation of thrombin, plasmin, FXa and FSAP. Whilst each can be activated from its zymogen form by CFHs, their respective activated forms can conversely deactivate or degrade CFHs [51, 71, 100]. Furthermore, the electrostatic nature by which CFHs manipulates haemostasis can also be modulated by the composition of charged molecules in its immediate milieu [33, 35, 54, 74], thus adding a further facet in predicting the procoagulant effect of CFHs. However, several clinical studies have shown concordance between antigenic and functional (cytotoxic) measurements of CFH in disease progression and prognostication.

Conclusion

The cell-based model was an advancement on the coagulation cascade model in refining haemostatic understanding and heralding therapeutic advances through replenishment or interference of pivotal coagulation effectors. Arguably, the added understanding of CFH involvement as well as that of other DAMPs can lead to further innovations, including in areas of unmet need where immunothrombotic problems are dominant and where therapies are limited.

Author Contributions

J. Yong, S.T. Abrams, G. Wang, and C.H. Toh contributed equally to the conceptualisation of this review. J. Yong reviewed the literature base and drafted the manuscript and figures. J. Yong, S.T. Abrams, G. Wang, and C.H. Toh contributed equally in revising and finalising the manuscript and figures for submission.

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Declaration of interests

JY, STA, GW, and CHT declare no conflict of interest.

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Figure legends

Figure 1. Cell free histone generation. A. Histones are released extracellularly as part of intact chromatin, either from cell death or NETosis. **B.** Extracellular degradation of chromatin by enzymes, including DNases-1 and -3, release nucleosomes. **C.** Nucleosomes dissociate into free DNA and histone octamers. Histone octamers further dissociate into histone dimers and finally into individual histones to exert their charge-dependent and charge-independent procoagulant effects. Free DNA can mediate charge-dependent procoagulant effects via the contact pathway. NETs degradation contribute histone- and DNA-independent procoagulant effects, e.g. through proteolysis of tissue factor pathway inhibitor (TFPI) by neutrophil elastase.

Figure 2. Cell-free histones in the initiation of coagulation. A. Histones disrupt the endothelial barrier through ionophore-induced apoptosis and gap junction disruption to enable circulating activated factor VII (FVII)a to access sub-endothelial tissue factor (TF) and generate TFase to activate factor X (FX). **B.** Histones induce intravascular TF expression by monocytes and endothelia through TLR-MyD88-AP1/NF-κB nuclear signalling pathways. TFase activity is enhanced by phosphatidylserine (PS) and protein disulphide isomerase mediated TF decryption, as well as FVIIa generation by histone-mediated autoactivation of FVII activating protease. **C.** Histones form an alternative prothrombinase complex by directly binding to FXa and generating thrombin without requiring phospholipid. Other abbreviations: TLR, toll-like receptor; AP1, activating protein-1; NF-κB, nuclear factor kappa B.

Figure 3. Cell-free histones in the amplification of coagulation. A. Histones activate, aggregate, and degranulate platelets via TLR signalling or by ionophore induction and calcium influx. Platelet degranulation contributes to procoagulant material bioavailability. **B.** Platelet polyphosphates (polyP) are released during platelet degranulation, which activates the intrinsic coagulation pathway. **C.** Histones induce endothelial degranulation via TLR signalling, which contributes ultra-large von Willebrand factor (VWF) towards local platelet recruitment. Other abbreviations: TLR, toll-like receptor.

Figure 4. Cell-free histones in the propagation of coagulation. A and B. Histones induce calcium influx through ionophore generation, activating scramblase which exposes phosphatidylserine (PS) on the outer surfaces of platelets and endothelia. Coupling of PS to the GLA-domains of the extrinsic tenase (activated factors IX and VIII) and conventional prothrombinase (activated factors V and X) complexes accelerates thrombin generation. **C.** Histones covalently bind fibrin clot via activated factor XIII transglutamination to confer fibrinolytic resistance. Histones also non-covalently associate with fibrin to form thicker, therefore more robust fibrin fibres. Other abbreviations: GLA, glutamic acid.

Figure 5. Cell-free histones in the reinforcement of coagulation. A. Histones inhibit the thrombomodulin (TM)-activated protein C (aPC) axis by binding directly to TM or zymogen protein C (PC). This reduces the aPC anticoagulant (cleavage of activated factors V and FVIII) and anti-inflammatory effects. **B.** Histones exert a parallel procoagulant effect via expression of inflammatory prostanoids, adherent receptors, and cytokines. **C.** Histones further consolidate clot formation by inducing neutrophil extracellular traps (NETs). NETs are procoagulant in physically juxtapositioning procoagulant components, including histones. Its integration enhances clot strength and viability through fibrinolytic resistance. NET degradation releases bound histones, adding to cell-free histones in circulation.