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Secretion of von Willebrand Factor and Suppression of ADAMTS-13 Activity by Markedly High Concentration of Ferritin

Dalia A. Bashir, MD[1,2], Qi Da, PhD[2,3], Subhashree Pradhan, PhD[2,3], Nitin Sekhar, MS[2,3], Christian Valladolid, PhD[2,3], Fong Lam, MD[1,2], Danielle Guffey, MS[4], Jordana Goldman, MD[1], Moreshwar S. Desai, MD[1], Miguel A. Cruz, PhD[1,2,3], Carl Allen, MD, PhD[1], Trung C. Nguyen, MD[1,2], and K. Vinod Vijayan, PhD[1,2,3]

Abstract

Hyperferritinemia is associated with poor outcomes in critically ill patients with sepsis, hemophagocytic lymphohistiocytosis (HLH), macrophage activation syndromes (MAS) and coronavirus disease 19 (COVID-19). Autopsies of hyperferritinemic patients that succumbed to either sepsis, HLH, MAS or COVID-19 have revealed disseminated microvascular thromboses with von Willebrand factor (VWF)-, platelets-, and/or fibrin-rich microthrombi. It is unknown whether high plasma ferritin concentration actively promotes microvascular thrombosis, or merely serves as a prognostic biomarker in these patients. Here, we show that secretion of VWF from human umbilical vein endothelial cells (HUVEC) is significantly enhanced by 100,000 ng/ml of recombinant ferritin heavy chain protein (FHC). Ferritin fraction that was isolated by size exclusion chromatography from the plasma of critically ill HLH patients promoted VWF secretion from HUVEC, compared to similar fraction from non-critically ill control plasma. Furthermore, recombinant FHC moderately suppressed the activity of VWF cleaving metalloprotease ADAMTS-13. These observations suggest that a state of marked hyperferritinemia could promote thrombosis and organ injury by inducing endothelial VWF secretion and reducing the ADAMTS-13 activity.

Keywords

disseminated intravascular thrombosis, endothelial dysfunction, sepsis, von Willebrand factor

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Introduction

Hyperferritinemia is associated with increased mortality in sepsis,[1-3] macrophage activating syndrome (MAS)[4] and hemophagocytic lymphohistiocytosis (HLH).[5,6] The current coronavirus disease-19 (COVID-19) pandemic also highlights the clinical relevance of hyperferritinemia because of its association with pathologic immune activation, increased severity of illness, disseminated microvascular thromboses, and mortality.[7-9] Importantly, there is a dose dependent relationship between the level of serum ferritin and mortality in septic and critically ill children.[2,10] Hyperferritinemia (> 500 ng/ml) has been considered as an acute phase reactant in clinical conditions with immune dysregulation.[3] However, emerging studies suggest that ferritin is a biologically active metabolite that can play a role in immune dysregulation and potentially result in organ injuries.[8,11,12]

[1] Department of Pediatrics, Texas Children's Hospital and Baylor College of Medicine, Houston, TX, USA
[2] Center for Translational Research on Inflammatory Diseases (CTRID), Michael E. DeBakey Veterans Affairs Medical Center (MEDVAMC), Houston, TX, USA
[3] Department of Medicine, Baylor College of Medicine, Houston, TX, USA
[4] Institute for Clinical and Translational Research, Baylor College of Medicine, Houston, TX, USA

Corresponding Authors:
Trung C. Nguyen, Texas Children's Hospital/Baylor College of Medicine, 6621 Fannin St, WT6-006, Houston, TX 77030, USA.
Email: trungn@bcm.edu

K. Vinod Vijayan, Baylor College of Medicine/MEDVAMC, Rm. 146, Bldg. 109, 2002 Holcombe Blvd, Houston, TX 77030, USA.
Email: vvijayan@bcm.edu

Ferritin is a multimeric iron storage protein with a spherical shell comprising of 24 subunits. These subunits are of 2 types namely, ferritin heavy chain (FHC) and ferritin light chain (FLC). FHC subunit contains 184 amino acids (~21 kDa), while FLC subunit has 174 amino acids (~19 kDa). The ratio of heavy to light chain subunit in a ferritin protein is tissue dependent.[13] Serum ferritin has high content of light chain,[14] is generally devoid of iron[15,16] and ranges from 10–300 ng/mL in normal individuals. Serum ferritin is mostly secreted by macrophages and hepatocytes[8,12,17] in response to interleukin-1 (IL-1), tumor necrosis factor-alpha (TNF-α) and interferon gamma (IF-γ).[17-21] Although ferritin is best understood for its role in iron metabolism, it also displays unique iron-independent function of influencing host immune response by paradoxically having both pro-and anti-inflammatory properties. For example, iron-free FHC can bind to specific T-cell immunoglobulin and mucin domain 2 (TIM-2) receptor on hepatic stellate cells and activate nuclear factor kappa B (NF-kB) pathway, resulting in an increased release of pro-inflammatory mediators such as IL-1β, inducible nitric oxide synthase, regulated on activation normal T cell expressed and secreted (RANTES) and intercellular adhesion molecules 1 (ICAM 1).[22] Conversely, ferritin can exert anti-inflammatory effects on host immune cells by decreasing granulocyte phagocytosis[23] and B lymphocyte antibody production.[24]

In addition to modulating immune cells, hyperferritinemia in critically ill children and adults is associated with coagulopathy and disseminated intravascular coagulation (DIC) in sepsis-induced multiple organ dysfunction syndrome (MODS) with MAS phenotype,[3] HLH,[6] and COVID-19 sepsis.[7,25-27] Previously, we reported that 42% of children, who died with HLH and hyperferritinemia, had disseminated microvascular thromboses. A majority (75%) of these children had evidence for both micro- and macro-vascular thromboses in the liver, kidneys, brain and heart on autopsy.[6] Importantly, in the same cohort, we found that median ferritin levels for non-survivors compared to survivors were 47,500 ng/mL (24,016-129,604 ng/mL) vs 11,884 ng/mL (3,130-36,602 ng/mL), respectively. In addition, DIC was present in 100% of non-survivors compared to 64% of survivors.[6] Similar to our data from the critically ill HLH children, an autopsy study of 67 adults who died with COVID-19 also showed features consistent with pathological immune activation such as hyperferritinemia, hemophagocytosis and disseminated microvascular thromboses.[25] Specifically, platelet-rich microthrombi were observed in pulmonary, hepatic and brain vasculatures.[25] Other COVID-19 autopsy studies also reported disseminated microvascular thromboses in multiple organs with platelet-rich and-VWF rich microthrombi.[28-30]

Thus, there is a strong association between hyperferritinemia, pathologic immune activation and disseminated microvascular thromboses. However, it is unknown whether VWF/platelet-rich microthrombi observed in clinical conditions of hyperferritinemia are a result of pathological immune activation or a direct effect of ferritin on vascular endothelium. In this study, we tested whether markedly high concentrations of ferritin observed in clinic can modulate the secretion of VWF and alter the activity of the plasma metalloprotease ADAMTS-13 (A disintegrin and metalloproteinase with thrombospondin type, member 13).

Materials and Methods

All human studies were performed with the informed consent from donors, which was approved by the Institutional Review Board of Baylor College of Medicine and Texas Children's Hospital.

Endothelial Cell Culture

Human umbilical vein endothelial cells (HUVECs) were obtained as previously described,[31] with minor modifications. Briefly, umbilical cord veins were washed with phosphate-buffered saline (PBS) and subjected to collagenase digestion. The resultant HUVECs were seeded onto 96 well culture plates and maintained in endothelial basal medium EBM-Plus (Lonza, Walkersville, MD, USA). The cells used in this study were from second or third passage.

Isolation of Ferritin Enriched Fraction From Human Plasma by Size Exclusion Chromatography

Blood from 5 critically ill HLH pediatric patients and 3 non-critically ill non-HLH patients was collected at the Texas Children's Hospital/Baylor College of Medicine, Houston. The median plasma ferritin level of HLH patient group was 41,800 ng/ml, while that for non-critically ill subjects was 162 ng/ml. Blood was centrifuged at 1600 rpm for 15 minutes to obtain plasma. Two-hundred microliters of plasma were diluted with 50 µl of Tris-buffered saline and the diluted samples were loaded onto size exclusion GE superose 6 columns (Amersham, Piscataway, NJ). Multiple 1 ml fractions were collected and, after initial characterization, plasma fraction #15 corresponding to molecular weight of ~21 kDa was identified to contain ferritin by immunoblotting with anti-ferritin heavy chain antibody ab75972 (Abcam, Cambridge, MA)

VWF Secretion Assay

Since ferritin concentration as high as 50,000 ng/ml to 100,000 ng/ml has been observed in HLH patients, we tested the effect of such markedly high concentration of ferritin on secretion of VWF by endothelial cells. HUVECs were stimulated with 50,000 ng/ml and 100,000 ng/ml of recombinant human ferritin heavy chain (FHC) protein (Abcam, Cambridge MA) in Opti-MEM media; 50,000 ng/ml and 100,000 ng/ml of synthetic human ferritin light chain (FLC) peptide (Abcam, Cambridge MA), and a combination of ferritin FHC and FLC peptide at a total concentration of either 50.000 ng/ml or 100,000 ng/ml for 30 minutes. Cells treated with only media (OptiMEM) served as negative control. To characterize the role and differences in biologic effects of endogenous ferritin derived from critically

ill patients with HLH and non-critically ill patients on secretion of VWF, 50 µl of the fraction #15 enriched in ferritin from both groups were also used to stimulate HUVEC for 30 minutes. In some studies, HUVECs were pretreated with 3 µg/ml of control antibody (rabbit isotype IgG) or 3 µg/ml anti ferritin antibodies (a combination of 1 µg/ml anti-ferritin light chain ab109019, 1 µg/ml anti-ferritin heavy chain ab75972 and 1 µg/ml of anti-ferritin antibodies ab75973 (Abcam, Cambridge MA) for 30 minutes, prior to stimulation with 50 µl of fraction # 15 from HLH plasma.

VWF in the cell supernatants was analyzed using ELISA as we have performed before.[32] Briefly, supernatant was incubated for 2 hours on microtiter plates pre-coated with 1 µg/ml rabbit anti-human VWF antibody (Dako, Santa Clara, CA). After several washes, 2 µg/ml of HRP conjugated anti-human VWF antibody (Dako, Santa Clara CA) was added and color developed with the addition of TMB (3, 3', 5, 5'-tetramethylbenzidine; Thermo Fisher Scientific, Waltham, CA). The reaction was stopped with 1 M HCl and the absorbance was read at 450 nm. Absorbance was converted to % of plasma VWF by comparing the absorbance values to a standard curve generated from pooled healthy human plasma samples. Fold differences in the VWF antigen levels were determined after normalizing the VWF values obtained for test samples (recombinant ferritin or ferritin isolated from patients) to that obtained from cell treated with OptiMEM media, which was set to a value of 1.

SDS-PAGE and Western Blotting

Plasma fractions #13, #14 and #15 obtained after chromatography of HLH plasma were separated on SDS-PAGE and immunoblotted with 2.5 ug/ml of anti-ferritin heavy chain antibody ab75972 (Abcam, Cambridge MA). Blots were developed using enhanced chemiluminescence system (Thermo-Scientific, Waltham, CA)) in a LI-COR odyssey Fc Imaging system (LI-COR, Lincoln, NE).

ADAMTS-13 Activity Inhibition

Blood was obtained from 3 healthy volunteers and centrifuged as above to obtain plasma. Plasma was incubated with recombinant FHC at 100,000 ng/ml (Abcam, Cambridge MA) or vehicle optiMEM control for 30 minutes. Activity of ADAMTS-13 in plasma after stimulation with FHC was detected with TECHNOZYM® ADAMTS-13 activity chromogenic ELISA kit (Diapharma, WestChester, OH).

Statistical Analysis

Continuous variables were summarized by means with standard deviation or median with 25th and 75th percentiles. Ferritin-induced VWF secretion was primarily expressed as a fold difference when compared with control (OptiMEM) samples. Comparisons of FHC, FLC, or FHC and FLC to control were analyzed using Wilcoxon signed rank test. Fraction #15 between critically ill (HLH) and non-critically ill (non-HLH) patients and the effect of ferritin antibodies was compared with Wilcoxon rank sum test. The change in ADAMTS-13 activity after adding FHC was assessed using generalized estimating equation (GEE) to account for the correlation within patients. Duplicate and triplicate analyses were averaged within patient for all analyses except GEE which statistically accounts for the correlation within patient. All statistical analyses were performed using Stata and Graph-Pad PRISM software was used for images. Differences were considered significant when $p < 0.05$.

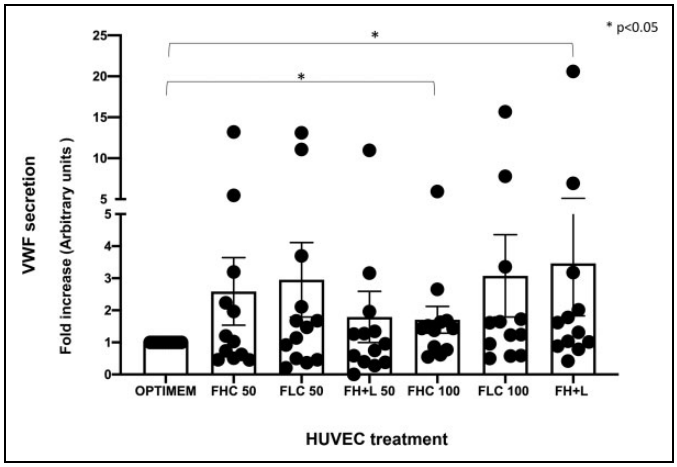

Figure 1. Recombinant ferritin induced VWF secretion. HUVEC were challenged with 50,000 and 100,000 ng/ml ferritin heavy chain peptide (FHC); 50,000 and 100,000 ng/ml of ferritin light chain protein (FLC); 50,000 and 100,000 ng/ml of combined FLC and FHC or media (OptiMEM). The supernatant was collected and the released VWF measured by ELISA. Fold increase in VWF secretion by FHC, FLC and its combination is relative to the VWF in the OptiMEM. N = 4 independent experiments, each performed in triplicates.

Results

Markedly High Ferritin Concentration Promoted VWF Secretion From Endothelial Cells

Although hyperferritinemia is one of the diagnostic markers in HLH, whether high ferritin level has a mechanistic or functional role remains unexplored. While the ratio of FHC to FLC is unknown, total ferritin concentration as high as 100,000 ng/ml has been observed in HLH patients and is associated with worse clinical outcome.[1] Since the release of VWF from endothelial cells support a prothrombotic milieu, we investigated if increasing doses of recombinant ferritin can induce VWF secretion from HUVECs. Compared to media, treatment of HUVECs with recombinant FHC, recombinant FLC peptide or a combination of FHC and FLC to a total concentration of 50,000 ng/ml did not significantly induce VWF secretion (Figure 1). Recombinant FHC at a concentration of 100,000 ng/ml significantly ($P < 0.05$) enhanced VWF secretion by 1.7 fold when compared to media (Figure 1). In contrast, recombinant FLC peptide at a concentration of

100,000 ng/ml did not significantly increase VWF secretion (Figure 1). Lastly, a combination of FHC and FLC at a total concentration of 100,000 ng/ml yielded ~3-fold increase in VWF secretion ($P < 0.05$); (Figure 1). These studies indicate that markedly high concentration of ferritin heavy chain or a combination of heavy and light chain at 100,000 ng/ml can facilitate *in vitro* secretion of VWF from HUVECs.

Ferritin Fraction Isolated From HLH Patient With Hyperferritinemia Promoted VWF Secretion

To provide a clinical relevance to the recombinant ferritin-induced secretion of VWF, we next assessed VWF secretion from HUVECs in response to endogenous ferritin enriched fraction obtained from the plasma of critically ill HLH and non-critically ill patients. Table 1 shows the ferritin levels in the critically ill and non-critically ill patient groups. Immunoblotting with anti-ferritin antibody revealed that fraction #14, fraction #15, but not fraction #13 from HLH plasma contained the ~21 kDa ferritin (Figure 2A). Compared to media, treatment with ferritin containing fraction #15 that was obtained from critically ill HLH patients significantly ($P < 0.05$) promoted ~3-fold increase in VWF secretion (Figure 2B). In contrast, stimulation of HUVECs with fraction #15 from the non-critically ill patients with lower ferritin level did not induce VWF secretion. The HLH fraction #15 mediated secretion of VWF from HUVECs was partially abrogated ($P < 0.05$) by anti-ferritin antibodies, but not with IgG control antibody (Figure 2C). These studies suggest an association between endogenous ferritin enriched fraction derived from the plasma of critically ill HLH patients and its potential to promote VWF secretion.

Table 1. Patient Diagnosis and Plasma Ferritin Levels.

Patient no	Diagnosis	Ferritin level (ng/ml)
	Critically ill with HLH symptoms	
1	Primary HLH	5770
2	Secondary HLH	12800
3	Secondary HLH	41800
4	Secondary HLH	58200
5	Secondary HLH	71500
	Non critically ill	
1	Familiar HLH (diagnosis made subsequent to blood draw)	221
2	Vertebral plana of T5	103
3	Giant cell granuloma	Not available

Recombinant FHC Reduced the Activity of ADAMTS-13 in Healthy Plasma

Endothelial cells secrete VWF multimer, which are hyper-adhesive for platelets and support a robust platelet/VWF-rich microthrombi. Such a strong prothrombotic outcome is normally kept in check by a metalloprotease ADAMTS-13 that cleaves VWF multimers. We tested if hyperferritinemia could affect the ability of ADAMTS-13 to cleave VWF. A modest, but significant decrease in the plasma ADAMTS-13 activity was observed in response to recombinant FHC at 100,000 ng/ml ($p < 0.05$) (Figure 3). These studies suggest that sustained hyperferritinemia has the potential to decrease ADAMTS-13 activity.

Discussion

Although hyperferritinemia and the prothrombotic phenotype coexist in certain clinical conditions, it is unknown whether high concentration of plasma ferritin can increase the risk of thrombosis. In this *in vitro* study, we demonstrate that very high concentration of ferritin can stimulate VWF release from the endothelium and suppress ADAMTS-13 activity. Thus, marked hyperferritinemia may contribute to a prothrombotic state via platelet/VWF hemostatic pathway.

Disseminated microvascular thrombosis and hyperferritinemia are often observed in critically ill patients suffering from pathologic immune activation, such as HLH, MAS, and

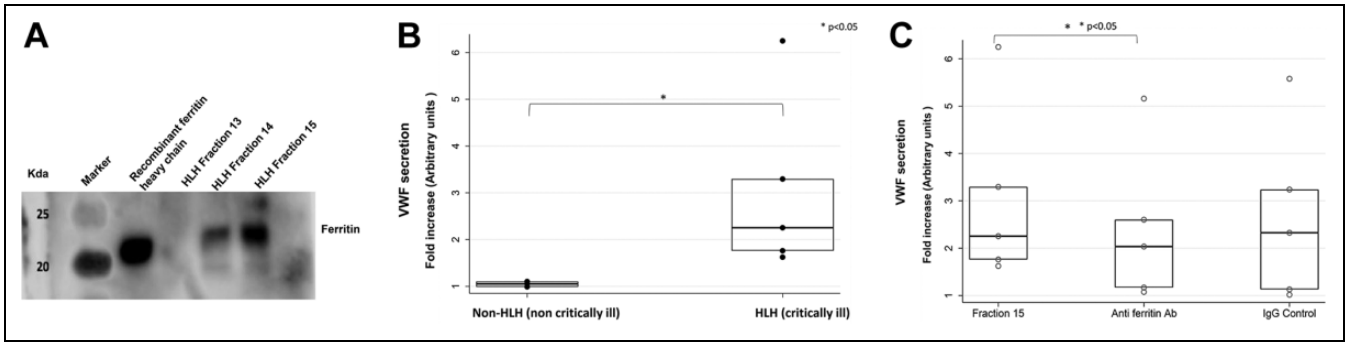

Figure 2. Ferritin enriched fraction isolated from HLH patients stimulate VWF secretion. (A) Following fractionation of HLH and non HLH plasma by size exclusion chromatography, fractions #13, #14 and #15 from HLH plasma were separated on SDS-PAGE gel and immunoblotted with anti-ferritin antibody. Recombinant ferritin is included as positive control. Blot is a representative of 5 experiments. (B) HUVECs was challenged with 50 μl of fraction #15 from 5 HLH patient and 3 non-HLH patient and secreted VWF measured using ELISA. (C) HUVECs were pretreated with either isotype IgG control antibody or anti-ferritin antibodies, prior to stimulation with fraction # 15 from 5 HLH plasma.

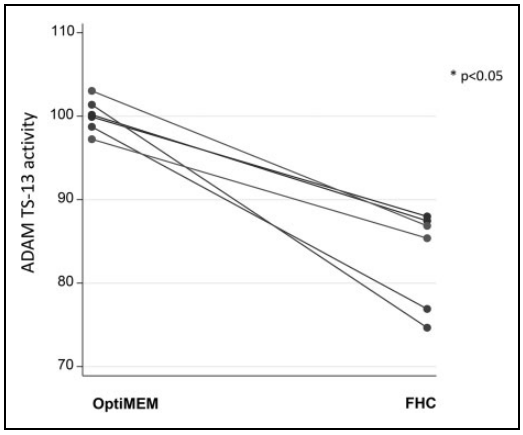

Figure 3. Recombinant FHC moderately decreased ADAMTS-13 activity. Plasma from 3 healthy subjects (shown in 3 different colors in duplicates) was mixed with either 100,000 mg/ml of FHC or media (OptiMEM) for 30 minutes prior to analyzing the ADAMTS-13 activity using an activity kit.

in COVID-19, all of which are caused by immune dysregulation.[6,25,28-30] Consistent with this observation, autopsy studies of these patients have revealed VWF-, platelet-, and fibrin-rich microthrombi in multiple organs.[6,25,28-30,33] However, the underpinning mechanisms that facilitate the formation of VWF/platelet-rich thrombi in these clinical conditions remain obscure. Since platelet and endothelial cells are critical players in microvascular thrombosis, we evaluated whether hyperferritinemia could alter platelet and endothelial physiology. Hyperferritinemia did not potentiate agonist-induced platelet aggregation or increase the expression of P-selectin, a platelet activation marker (not shown), suggesting that platelets are unlikely to be impacted directly by hyperferritinemia. In contrast, our studies showed that ferritin at markedly high concentration (100,000 ng/ml but not 50.000 ng/ml) supported VWF secretion from endothelial cells. Specifically, recombinant ferritin heavy chain but not ferritin light chain at 100,000 ng/ml promoted VWF secretion (Figure 1). Interestingly, although ferritin light chain by itself did not support VWF secretion, when combined with ferritin heavy chain, there was a notable synergistic effect [1.7 fold (FHC) to 3 fold (FHC+FLC)] on VWF secretion. Moreover, endogenous ferritin containing fraction from critically-ill HLH patient plasma also promoted VWF secretion (Figure 2).

What is the significance of marked hyperferritinemia-induced VWF secretion and suppression of ADAMTS-13 activity? During systemic inflammation, endothelial cells of the vascular bed respond to the inflammatory cytokines by secreting thrombotic and inflammatory modulators, including prothrombotic VWF multimers via the inducible pathway that can cause spontaneous platelet aggregation and promote thrombosis.[34,35] Upon release, these VWF multimers are rapidly, but partially cleaved by a plasma metalloprotease ADAMTS-13.[35] We observed increased VWF secretion and moderate but significant suppression of plasma ADAMTS-13 activity by high ferritin concentration (Figures 1 and 3). Thus, marked hyperferritinemia-induced VWF secretion and partial attenuation of ADAMTS-13 activity could render patients at a greater risk for developing disseminated microvascular VWF/platelet-rich microthrombi. Consistent with this argument, we previously reported that thrombocytopenia-associated multiple organ failure (TAMOF) patients with severe sepsis have elevated VWF activities and acquired partial ADAMTS-13 deficiency, with autopsy findings of disseminated microvascular thrombosis.[33] More recently, 2 large multicenter studies have also validated TAMOF as a distinct sepsis-induced MODS phenotype with ADAMTS-13 deficiency and high mortality.[3,36] Besides the thrombotic implications, marked hyperferritinemia-induced VWF secretion can also sustain the inflammatory milieu. Secreted VWF can enable the recruitment of leukocytes directly via β2 integrins or p-selectin glycoprotein ligand-1 (PSGL-1) to the endothelium, or indirectly via an interaction with platelet GPIb.[37] Furthermore, VWF can alter the vascular permeability and incorporate into the neutrophil extracellular traps (NETS), structures formed from DNA and histone released by activated neutrophils during inflammation and potentiate thrombosis.[38,39]

Although marked hyperferritinemia is capable of modulating VWF secretion *in vitro*, it is unlikely that high ferritin concentration in isolation can cause disseminated microvascular thrombosis. Future studies will examine if a combination of ferritin and inflammatory mediators including acute phase reactant such as C reactive protein (CRP) could trigger thrombotic risk. Indeed, increased risk for thrombosis was not observed in patients with hyperferritinemia-cataract syndrome caused by a mutation in the iron response element region.[40] Pathologic immune activation and coagulopathy are intimately intertwined as activated immune cells and endothelium can initiate coagulation through tissue factor synthesis, besides altering other coagulation mediators.[41]

There are several limitations in this study. Although, hyperferritinemia is documented in critically-ill HLH patients, we have modeled ferritin concentration at the very high end in these studies. It remains to be studied whether ferritin concentrations less than 100,000 ng/ml along with a secondary stimulus can modulate VWF secretion and alter ADAMTS-13 activity. Since we have only isolated a plasma fraction enriched in ferritin and not purified endogenous ferritin from the HLH plasma, the potential effect of other likely proteins within the fraction, including ferritin binding proteins (apolipoprotein B and fibrinogen) on VWF secretion cannot be excluded. Despite these concerns, fraction #15 from non-critically ill patients did not support VWF secretion. Moreover, the ability of fraction #15 from critically-ill HLH patients to promote VWF secretion was partially but significantly reduced by anti-ferritin antibodies. We cannot rule out that serum-iron levels are elevated in HLH patients with hyperferritinemia, as unliganded iron has been shown to cause a prothrombotic state.[42]

In conclusion, we show that markedly high ferritin concentration can promote secretion of VWF from the endothelial cells and can partially suppress ADAMTS-13 activity.

Moreover, endogenous plasma fraction enriched in ferritin obtained from critically-ill HLH patients with documented hyperferritinemia can also facilitate VWF secretion. The observation that marked hyperferritinemia-induced elevation of VWF and partial suppression of ADAMTS-13 activity has the potential to generate new hypothesis for future studies with pathological immune activation. The concept that marked hyperferritinemia is a mediator of pathologic immune activation and thrombosis may open new avenues for ferritin targeted therapies in patients with hyperferritinemia and pathologic immune activation.

Authors' Note

DAB, QD, SP, NS, CV performed the studies and obtained data. FL and MSD analyzed studies and edited the manuscript. GD is a statistician and performed statistical analysis. JG, CA provided the clinical samples. MAC, TCN and KVV designed the study, analyzed data and edited the manuscript. Written informed consent was obtained from legally authorized representative(s) for anonymized patient information to be published in this article. The content of this article does not represent the views of the Department of Veterans Affairs or the US Government.

Declaration of Conflicting Interests

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