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Review Article

Hemoglobin Mediated Regulation of Platelet Functions

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Abstract

In patients of hemolytic disorders, presence of excessive free hemoglobin (Hb) in plasma causes several cytotoxic effects. Hb being a potent scavenger of nitric oxide (NO) impairs the NO-mediated vasodilatory functions, thus promoting blood vessel constriction and related clinical events in hemolytic patients. This decrease in endogenous level of NO, an inhibitor of platelet activation, increases thrombophilic complications in these patients. Hb also generates reactive oxygen species (ROS) and affects several cellular functions. Hypercoagulation, thrombosis and inflammation are hallmark features of hemolytic disorders like sickle cell disease (SCD), paroxysmal nocturnal hemoglobinuria (PNH), thalassemia, hemolytic uremic syndrome (HUS) and Aplastic anemia (AA). We have recently described a novel mechanism of Hb mediated activation of platelets. We have shown that Hb binding to glycoprotein (GP)-1b alpha on platelet, leads to platelet activation and binding to Von Willebrand factor (VWF) increases the VWF-platelet binding, promoting thrombus formation. Herein, we will briefly discuss the role of Hb in modulating the platelet functions in the backdrop of pathophysiological conditions like hemolytic disorders including PNH and SCD.

ABBREVIATIONS

Hb: Hemoglobin; SCD: Sickle Cell Disease; PNH: Paroxysmal Nocturnal Hemoglobinuria; Hp: Haptoglobin; VWF: Von Willebrand Factor

INTRODUCTION

Hemoglobin: pathophysiological roles

Hemoglobin (Hb) is a molecule that has remained highly conserved across species and is encapsulated within erythrocytes. It primarily serves the respiratory system in mammals by transporting oxygen to tissues from lungs and removing carbon dioxide [1,2]. Hb is released into the circulation from erythrocytes as a result of intravascular hemolysis in acquired, hereditary or iatrogenic hemolytic conditions. Although it plays a vital role as a carrier of oxygen, Hb is highly toxic in its unbound, cell-free state [1-6]. To neutralize the toxicity of cell-free Hb, it is cleared from the plasma by specialized scavenger proteins-haptoglobin, CD163 and hemopexin. After the release of Hb into the plasma, it dimerizes and binds to haptoglobin (Hp) to make haptoglobin-hemoglobin (Hp-Hb) complex. This Hp-Hb complex further interacts with macrophage receptor CD163, resulting in its endocytosis and subsequent degradation. Simultaneously, Hb present in plasma upon oxidation releases ferric heme, which is bound by hemopexin (Hpx) and degraded in the liver by hepatocytes [1,3-5,7-9]. Cell-free Hb depletes Nitric oxide (NO) by reacting with it irreversibly and generates hydroxyl radicals which initiate membrane lipid peroxidation ultimately leading to cellular damage. Limiting the bioavailability of NO by cell-free Hb disrupts NO-dependent vasomotor function of the endothelia thus leading to endothelial dysfunction and multiple organ failure. Cell-free Hb produces superoxide anions through pseudoperoxidase (POX) activity, which further reacts with hydrogen peroxide along with heme. This drives the peroxidase and Fenton reactions, thus generating ferryl heme and hydroxyl radicals. Thus, cell-free Hb and Heme induces oxidative stress by taking part in the production of reactive oxygen species (ROS), leading to lipid peroxidation and cellular damage.

Hemoglobin: impaired coagulation and thrombotic events

Intravascular hemolysis in hemolytic disorders such as in PNH and SCD, results in a huge buildup of Hb in the plasma, which leads to decreased response of detoxifying systems. Elevated levels of Hb in the plasma causes vascular and organ dysfunction which leads to adverse clinical signs and symptoms .Hemolytic disorders like PNH and SCD, which are characterized by intravascular hemolysis, are always associated with hyper coagulable states [3,4,10-17]. The cell-free Hb released during hemolysis generates ROS, subsequently leading to activation of platelets [18]. It also scavenges NO, which is essential for regulating smooth muscle tone. As NO plays an important beneficial role in vascular

homeostasis, its depletion decreases the activation of guanylate cyclase, which in turn reduces the production of cyclic guanine monophosphate (cGMP), resulting in endothelial dysfunction and vasoconstriction, further leading to dystonia, hypertension, and dysphagia. Reduced bioavailability of NO also triggers platelet activation, aggregation and promotes clot formation [1,2,5,11,19]. Platelets are the first cellular corpuscles which are recruited at sites of vascular injury to stop bleeding by forming a platelet plug. Platelets have variety of receptors on their surface among which Glycoprotein (GP)1bα and integrin αIIbβ3 play key role in platelet activation and aggregation [20,21]. During vascular injury, platelets interact with von Willebrand factor (VWF) through GP1bα and trigger intracellular signaling events, such as activation of protein kinase pathways and elevation of calcium levels in cytoplasm, which result in platelet activation. GP1bα-triggered intracellular signaling leads to activation of ligand binding function of αIIbβ3 and binding to fibrinogen, thus promotes thrombus formation in order to form a platelet plug, which is a homeostatic function [17,20-22]. Recent studies reveal that in hemolytic disorders, such as PNH, Hb directly interacts with GP1bα on platelet surface, activates platelets and also promotes platelet apoptosis by initiating GP1bα mediated intracellular signaling [17, 22, 23].

Intravascular hemolysis is one of the primary phenomena observed in a number of hemolytic diseases like PNH, hemolytic uremic syndrome (HUS), thalassemia and hemolytic anemia. During intravascular hemolysis, extracellular hemoglobin (Hb) triggers several pathophysiological events which are associated with clinically undesirable outcomes, such as hyper coagulation, thrombosis, inflammation, vascular problems, and abnormalities associated with the urinary system. PNH is one such prototypic intravascular hemolytic disorder in which excessive release of hemoglobin in plasma is toxic, leading to subsequent platelet activation and uncontrolled complement activity and hence systemic complications. Hemoglobinuria (the hallmark of PNH) leads to manifestation of intravascular hemolysis and is associated with various clinical abnormalities like abdominal pain, erectile dysfunction, thrombosis and fatigue [24-26]. SCD is another hemolytic disease which is characterized by sickle shaped red blood cells, chronic intravascular hemolysis and high propensity to vaso-occlusive crisis [27,28]. Besides, other clinical complications such as hypercoagulation and thrombosis are considered as leading causes of morbidity in these patients [1,29]. Intravascular hemolysis i.e. abnormal breakdown of RBCs in blood vessels contributes to the pathogenesis of thrombosis and thromboembolism in hemolytic disease conditions. In hemolytic conditions, excessive hemoglobin (Hb) is released into the extracellular fluid and ensues into several toxic effects on cellular functions including thrombosis and hypercoagulation. A number of report also conclude that intravascular hemolysis during PNH contributes primarily to thrombotic disorders and thromboembolic events [30].

Hemoglobin: impaired platelet functions

Platelet activation involves a series of signalling events involving various kinases, which enable platelet recruitment, stable platelet adhesion and thrombus stabilization. At vascular injury site, the platelets adhere to the exposed endothelial cells through collagen- GPVI, VWF-GPIb-IX-V and fibronectin- integrin α5β1coupling. Platelets adhere to collagen and adopt an active conformation. The various signalling pathways associated with receptor-specific platelet activation converge into common signalling nodes that stimulate change in shape of platelets and secretion of granules which lead to "inside-out" signalling, that induces activation of the ligand-binding function of integrin $\alpha_{IIb}\beta_3$. Interestingly, despite considerable differences in their structure and functions, majority of platelet adhesion and activation receptors have several signalling pathways in common. For example, signal transduction through the glycoprotein Ib-IX-V complex (GPIb-IX), GPVI and integrins all involve Src family kinases (SFKs), phosphoinositide 3-kinases (PI3Ks, and the immune receptor tyrosine-based activation motif (ITAM). Activated platelets secrete ADP, platelet-derived growth factor and fibrinogen from their storage granules and thromboxane A_2 (TxA_2) is produced by immediate biosynthesis. ADP and TxA_2 cause circulating platelets to change their shape. Glycoprotein IIb/IIIa receptors on the surface of activated platelets binds fibrinogen, that triggers "outside-in" signalling. This facilitates the formation of fibrinogen bridges between platelets and ultimately platelet aggregation. Simultaneously, fibrin mesh develops which gives rise to platelet thrombus. It is followed by clot retraction that leads to the formation of a stable thrombus [31].

Platelets adhere to collagen or thrombin, which triggers the procoagulant response. In later stages of activation, platelets initiate the blood coagulation pathway by providing a surface where the coagulation factors bind and are activated to generate thrombin. Besides, providing the procoagulant site and binding surface sites for various coagulation and thrombotic molecules, platelets contribute to coagulation activity by releasing several factors, such as FV, FXIII, fibrinogen, VWF and protein S, which play significant role in hemostasis [31]. NO scavenging mediates the major effects of plasma Hb on platelet functions. NO has been shown to be associated with inhibition of platelet activation and initiation of disaggregation of platelet aggregates and it also increases cGMP levels to inhibit platelet adhesion [32]. Toxicity of the cell-free Hb and depletion of NO has been implicated in the initiation of platelet activation and aggregation [1]. It leads to the release of ADP by RBCs [33-35]. Once in the blood stream, ADP can cause platelet activation. A reversible platelet aggregation has been shown upon ADP infusions in rats and rabbits [36,37]. There are studies in rats demonstrating administration of Hb associated with various molecules, leading to increased platelet aggregation and adhesion *in vivo* on prothrombotic surfaces such as an injured vessel wall. Phosphatidylserine (PS) bearing RBCs are more prone to endothelium adherence. They provide a possible structural binding site for complexes such as tenase and prothrombinase [38]. Furthermore, *ex vivo* studies of blood from transfusion recipients have shown increased platelet activation and aggregation attributed to ADP released from red blood cells [39]. It has been earlier reported that Hb scavenges NO and also reduces its generation by inhibiting metabolism of arginine that consequently leads to increase in platelet aggregation [40,41]. Furthermore, the externalization of PS and production of micro particles is also recognized in platelets that may act as a binding site for complexes like prothrombinase and tenase [25,31].

Hemolytic diseases are often associated with

hypercoagulability and increased platelet activation [42-44]. Villagra et al., reported a correlation between platelet activation and markers of hemolysis in SCD [13]. A high number of cases associated with thrombotic complications have been reported in different hemolytic anemias (HA) (Figure 1), particularly in SCD [45-47], thalassemia [48,49] and PNH [10]. Pathophysiology in all of these aforementioned diseased conditions are different but all have a common procoagulant condition and also they often share potential hemolysis related sequelae that include pulmonary and systolic arterial hypertension, cutaneous leg ulcerations, postsplenectomy, thrombosis and possibly stroke [1,50]. Several mechanisms have been proposed to be involved in these events, comprising anomalous RBC properties, increased number of micro particles in plasma, excessive cell-free Hb resulting in reduced NO bioavailability, increased concentration of oxidants in the blood and endothelial dysfunction [14]. Apart from these indirect associations of Hb with platelets, recent research focuses on direct binding of Hb with platelet receptor

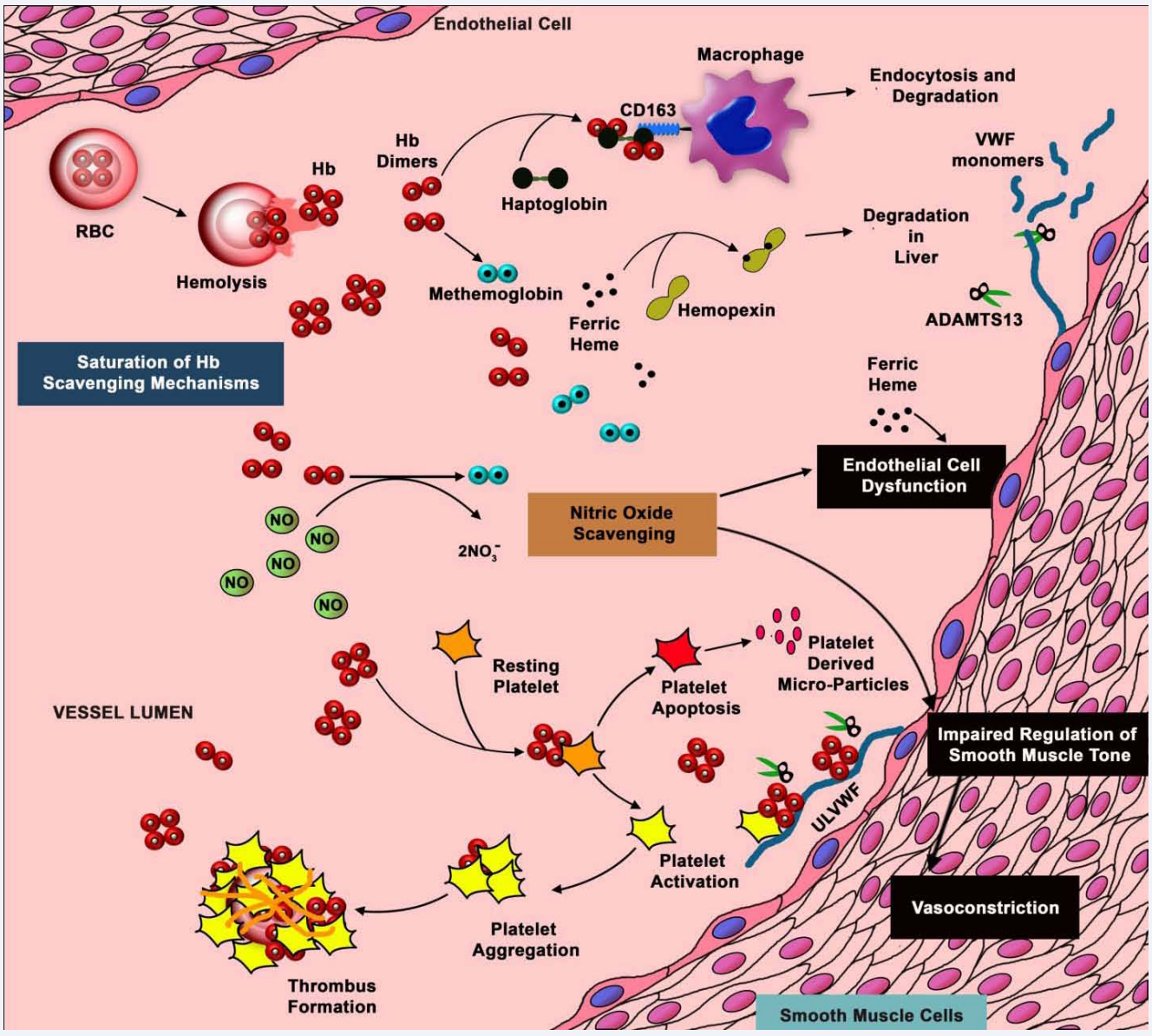

Figure 1 Schematic elucidation of role of hemoglobin in regulation of platelet functions. Intravascular hemolysis leads to release of Hb into the plasma, where it is routinely cleared by haptoglobin, CD163, and hemopexin which act as Hb scavengers. Haptoglobin forms complex with Hb and this complex get endocytosed and eventually degraded by macrophages/monocytes via CD163 binding. Hemopexin binds to ferric heme which is released upon oxidation of Hb and degraded by hepatocytes. Saturation and depletion of the above Hb scavenging systems instigate build-up of Hb and heme. Plasma hemoglobin and heme can impair regulation of smooth muscle tone either by causing direct pro inflammatory and pro-oxidant effects on vessel endothelial cells via ferric heme or indirectly by NO scavenging. The cell free Hb can also induce platelet activation. It binds directly to VWF, blocks ADAMTS13 mediated proteolysis and increases the affinity of the VWF A1 domain for the glycoprotein Ib (GPIb) receptor on the surface of platelets. The increased interaction between platelets and Hb then allows a massive binding of platelets to insolubilized fibrinogen or to components of the extracellular matrix such as collagen which ultimately leads to thrombus formation.

GP1bα and regulating platelet functions.

Hb binding to GP1b and platelet activation

Recent studies revealed that Hb binds to the platelet receptor GP1bα (at N-terminal) and triggers the platelet activation. Thus platelet activation by Hb could also be attributed to its direct interaction with platelet GP1bα and activation of downstream signalling events. Hb-GP1bα interaction stimulates events such as platelet shape change, granule secretion and inside-out signalling process leading to activation of the ligand binding function of integrin GPIIbIIIa [17]. Lyn/PI3K/Akt/NO/cGMP/PKG/MAPK pathway reportedly plays an important role in GPIbα mediated platelet activation [31]. Supporting this, Singhal et al., have shown that Hb binding to platelet receptor GP1bα induces the inside-out signalling through this pathway and blocking this interaction by specific peptide (designed against N-terminal region of GP1bα) attenuates or completely switches off the GP1bα associated molecular events initiated by Hb [17]. Further, morphological change in the structure of GPIIbIIIa is an important prerequisite for platelet activation. Shattil et al., developed and characterized a murine monoclonal anti-platelet antibody, designated as PAC-1 that binds to activated platelets i.e, the open conformation of GPIIbIIIa. Platelet activation appears to cause a change involving the glycoprotein IIbIIIa complex that exposes the fibrinogen receptor and at the same time, the epitope for PAC-1 [51]. Singhal et al., have shown that Hb increased the activation of platelet receptor GPIIbIIIa in a concentration-dependent manner up to a concentration of 6μ Mas evidenced from the binding of PAC1-FITC antibody to platelet GPIIbIIIa in presence of Hb [17].

Hb binding to VWF enhances platelet adhesion and activation

Hb acts as an additional bridge between VWF and GP1bα and significantly augments and potentiates the interaction of VWF with its receptor GP1bα on platelet. It has been shown that free Hb binds directly to VWF, thereby increasing the affinity of the VWF A1 domain for the glycoprotein Ib (GPIb) receptor on the surface of platelets. The increased interaction between platelets and VWF then allows a massive binding of platelets to insolubilized fibrinogen or to components of the extracellular matrix such as collagen [52]. This alteration in platelet binding and aggregation induced by free Hb may contribute to the development of thrombi. Recently, Hemoglobinemia has been proposed as a possible causative factor which contributes to clinical manifestations in thrombotic microangiopathies (TMAs), which is characterized by microangiopathic hemolytic anemia and thrombocytopenia [53]. For several years it has been recognized that VWF has an important role in the pathogenesis of TMAs. VWF mediates the adhesion of platelets to sites of vascular injury and as the carrier protein for coagulation factor VIII. It is also required for factor VIII survival in the circulation. High shear stress conditions give rise to ultra Large multimers of VWF (ULVWF) which is the most biologically active form in platelet-vessel wall interactions and directly induce platelet adhesion and aggregation [54].There are reports which suggest that extracellular Hb addition to serum decreases the activity of enzyme ADAMTS13, which is a metalloprotease and is important in limiting VWF activity and preventing formation of platelet thrombi [55-57]. It has been reported that SCD patients have increased levels of ULVWF multimers in their plasma, though they have a very mild or no significant deficiency in ADAMTS13 activity [56,58,59]. ULVWF multimers freshly secreted from endothelial cells accumulate, if not properly cleaved by ADAMTS13, leading to severe thrombotic microangiopathy [60-62]. Cell free Hb interacts with VWF to inhibit its cleavage by ADAMTS13, and the mechanism is independent of metalloprotease activity [56]. It binds to the ADAMTS13 cleavage site on the A2-domain of the VWF multimer to block the VWF cleavage by the metalloprotease. The high molecular weight, ULVWF multimers coexists with high plasma level of cell free Hb in SCD patients [56,57,63,64]. These multimers are hyper reactive compared to normal individuals [64]. The presence of ULVWF is a prime requirement for the formation of platelet thrombi. ULVWF multimeric strings are anchored to the endothelial cells via P-selectin molecules present on platelet surface. Platelets adhere through GPIbα to the ULVWF multimeric strings anchored to P-selectin, which further favour platelet aggregation under flow conditions. This forms large, potentially occlusive platelet thrombi. In addition to platelet aggregation and ADAMTS13 deficiency, intense hemolysis and NO consumption link TMA to other hemolytic conditions and this explains the wide spectrum of clinical manifestations associated with this condition.

Hb mediated platelet apoptosis and necrosis

Anucleated platelets have the capacity to undergo programmed cell death [65]. Many of the features of apoptosis (membrane fragmentation, cytoskeletal disruption, microvesiculation, caspase activation and PS exposure) are observed during prolonged platelet storage *ex vivo* and during the conversion of activated platelets to a procoagulant state, raising the possibility that apoptosis may also regulate platelet function [66,67]. A fundamental, but incompletely understood aspect of platelet function is the relationship between a regulated, naturally occurring platelet activation response, i.e., platelet procoagulant function and the cell death pathways modulating platelet survival. In this context, procoagulant platelets are not just highly activated cells, they also undergo cell death. They have all the biochemical, morphological and functional features of a dying cell, including caspase and calpain activation, proteolytic processing of cytoskeletal elements, surface exposure of PS, membrane contraction, blebbing and microvesiculation [68]. Morphologically, procoagulant platelets lose their internal organelles and cytoskeletal integrity, and functionally these platelets have lose their ability to adhere and aggregate with other platelets [69]. Although the role of platelet activation in hemostasis and thrombosis is well-documented, the role of platelet apoptosis in these vital processes and of platelet apoptosis versus activation in platelet clearance is still to be elucidated. Recent reports suggest that high concentration of Hb regulates platelet apoptosis [17]. Platelets undergoing apoptotic process are procoagulant in nature and induced to become so through a Bak/Bax-mediated apoptotic pathway. Bak/Bax form pores within the outer mitochondrial membrane, which leads to release of cytochrome c (CytC) from the mitochondrial inner membrane that involves initiator caspase and executioner caspase activation. This ensures exposure of PS which provides a major clearance signal for phagocytes. This process is

independent of platelet activation and release of granules [68]. A recent study has shown that Hb induces caspase pathways, expression of proapoptotic proteins, cytochrome C release and PS externalization in platelets. Study also elucidated the procoagulant function of platelets in response to extracellular Hb stimulation during platelet apoptosis by thrombin generation [17].The link of platelet apoptosis and released microparticles (MPs) from platelets has been known from before. Platelet-derived MPs are sub-cellular and circulating vesicles that are released during platelet activation or apoptosis, ranging in size from 0.1 μm to 1.0 μm (21). High level of Hb-bound platelets/platelet MPs existed in circulation of patients with hemolytic diseases such as PNH and SCD [17].

Apoptotic cell death is a physiological process essential for the removal of unwanted, aged or damaged cells without any accompanying inflammatory response. In contrast, the necrotic cell death is associated with loss of plasma membrane integrity and the consequent release of cellular contents into the extracellular environment. While traditionally viewed as an unregulated by-product of severe pathologic injury, increasing evidence suggests that necrotic cells can elicit controlled biological responses that are important for host defense and repair [69].

The initiation of necrotic platelet death at sites of vascular injury may play an important role in inducing inflammatory and repair processes. Agonist-induced procoagulant platelets play a central role in promoting thrombin generation and the development of the 3-dimensional fibrin matrix [70]. Furthermore, procoagulant platelets produce high levels of the proinflammatory lipid, Platelet Activating Factor (PAF) and have enhanced reactivity toward neutrophils, raising the possibility that they play an important physiological role in promoting leukocyte recruitment/activation at sites of vascular injury [71]. These functional properties of procoagulant platelets are broadly consistent with the role of cell necrosis in physiological inflammatory and repair responses. The contribution of necrotic platelet population in supporting thrombotic events during hemolytic disorders remains to be elucidated.

CONCLUSION AND FUTURE PROSPECTIVE

Intravascular hemolysis contributes to the pathogenesis of thrombosis and thromboembolism. In hemolytic disease conditions, excessive extracellular Hb is released and causes a plethora of toxic effects on cellular functions including thrombosis and hypercoagulation. The knowledge regarding the effect of extracellular Hb on various platelet functions, particularly in hemolytic diseases continue to emerge. We have focused on role of Hb in regulating platelet functions such as adhesion, aggregation, activation, apoptosis and microvesiculation in PNH and SCD. Thus, it seems there is substantial scope of developing novel tools, such as receptor antagonists or recombinant peptides that specially interfere in the Hb induced activation of platelets. Thus, these approaches hold promise for the reduced risk of Hb associated thrombotic complications.

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