Heme: Modulator of Plasma Systems in Hemolytic Diseases

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Hemolytic diseases such as sickle-cell disease, β-thalassemia, malaria, and autoimmune hemolytic anemia continue to present serious clinical hurdles. In these diseases, lysis of erythrocytes causes the release of hemoglobin and heme into plasma. Extracellular heme has strong proinflammatory potential and activates immune cells and endothelium, thus contributing to disease pathogenesis. Recent studies have revealed that heme can interfere with the function of plasma effector systems such as the coagulation and complement cascades, in addition to the activity of immunoglobulins. Any perturbation in such functions may have severe pathological consequences. In this review we analyze heme interactions with coagulation, complement, and immunoglobulins. Deciphering such interactions to better understand the complex pathogenesis of hemolytic diseases is pivotal.

Pathophysiological Potential of Heme

Heme (see Glossary) is a complex of protoporphyrin IX with an iron ion (Box 1). It is an essential cofactor molecule in all forms of aerobic life. In addition to its prominent functions in oxidative metabolism, heme is involved in the regulation of important cellular processes such as gene expression, translation, and the cell cycle [1–5]. Under physiological conditions, heme is sequestered intracellularly and is tightly bound to the polypeptide chains of different hemo-proteins. However, as a result of various pathologies, including hemolytic diseases, thrombotic microangiopathies, subarachnoid hemorrhage, rhabdomyolysis and infectious diseases (such as malaria), large amounts of hemoproteins may be released into plasma [6,7]. In cases of congenital hemoglobinopathies such as sickle cell disease, deficiencies in complement system regulators such as paroxysmal nocturnal hemoglobinuria, and many other disorders, erythrocytes can lyse and liberate large quantities of hemoglobin. In vitro experiments demonstrate that the reduced status of heme iron in extracellular hemoglobin is difficult to maintain and that heme is easily transformed into its oxidized (Fe³⁺) form and released from the protein complex [8,9] (Boxes 1–3). Extracellular hemoglobin and heme are pro-oxidative, proinflammatory, and cytotoxic [10–12], and can contribute to the pathology of hemolytic diseases. These noxious activities explain the presence of proteins that scavenge hemoglobin and heme in plasma [7,13,14]. Thus, the interactions of hemoglobin with haptoglobin, and of heme with hemopexin, ensure safe disposal of potentially dangerous molecules [6,7,15–19] (Boxes 1–3). In cases of extensive and chronic hemolysis, levels of haptoglobin and hemopexin in plasma decrease markedly [20,21]. Accordingly, high concentrations of heme (20–50 μM) in the plasma of patients with severe hemolytic events may be achieved [20]. Excess extracellular heme is weakly bound to albumin [22], α1-microglobulin (which can also degrade heme) [23] and, most likely, to other plasma proteins and lipoproteins; there is a dynamic nature to such
Box 1. Generation of Hemolysis-Derived Products

Hemoglobin (Hb) and Heme

Erythrocytes contain Hb, which plays an essential role as a gas carrier in different organisms. This 64 kDa protein is made of four globin chains (two α and two β), each bound to a prosthetic group – heme. Heme is a ubiquitous iron-containing compound, present in large amounts in all animal cells. During hemolysis, erythrocyte destruction leads to Hb release into the circulation. Tissue injury, inflammation, sepsis, subarachnoid hemorrhage, or atherosclerosis are associated with local Hb release. In the extracellular milieu, Hb undergoes different oxidation stages resulting in the release of free heme. Recent updates on the Hb oxidation cascade have been described elsewhere [18].

Hb Oxidation, Heme Release, and Scavenging Systems

The first form of Hb released from lysed red blood cells is oxygenated ferrous Hb(Fe²⁺−O₂). The presence of HbFe³⁺−O₂ in blood vessels triggers depletion of nitric oxide (NO) – a physiological vasodilator, thus leading to vessel vasoconstriction and a hypertensive response. Auto-oxidation and reaction with NO and other oxidants convert HbFe³⁺−O₂ to ferric Hb (Fe³⁺), also called methemoglobin. A further oxidative processes leads to formation of ferrylHb(Fe⁴⁺), which is unstable and returns to the Fe²⁺ state by reacting with specific amino acids of the globin chains. The oxidized forms of Hb bind with very high affinity (Kd < 10⁻¹⁵ M) to haptoglobin; the complex is endocytosed by macrophages through CD163 engagement. Haptoglobin, however, can be rapidly saturated. Indeed, the endogenous concentration of haptoglobin in humans is about 1–3 mg/ml sufficient to neutralize only about 0.5% of the total erythrocyte Hb [19]. Excess ferric Hb(Fe³⁺) results in the release of free heme in the circulation or tissue microenvironment, and may be scavenged by hemopexin. Hemopexin is a very efficient heme scavenger and the complex forms with very high affinity (Kd < 10⁻¹³ M), rendering the association virtually irreversible [7]. The complexes are recognized and endocytosed by CD91 expressed by macrophages and hepatocytes, where a part of hemopexin is recycled, heme is degraded, and its iron reutilized [7]. When hemopexin is saturated by excessive heme release, heme binds loosely to other proteins, including albumin and α1-microglobulin (as well as to immunoglobulins, complement, coagulation proteins) [23,106], and lipids [107]. It can also penetrate inside cells, driving the oxidation of unsaturated fatty acids, triggering inflammation, and causing cell and tissue damage.

Box 2. Degradation of Hemolysis-Derived Products

Heme Degradation

Within cells, heme induces the expression of heme oxygenase 1 (HO-1), a cytoprotective enzyme which metabolizes heme to biliverdin, carbon monoxide (CO), and iron [25]. Biliverdin is metabolized to bilirubin by biliverdin reductase. Bilirubin and CO have anti-inflammatory, antioxidant, and anti-apoptotic properties and may counteract heme toxicity. Nevertheless, in cases of major oxidative stress, bilirubin and biliverdin are further degraded by free radicals into bilirubin oxidation end-products (BOXes) that have potent adverse vasocconstriction properties [106]. Of note, α1-microglobulin can also degrade heme in the extracellular milieu, with consequences that are not well understood [23,106,109].

Box 4. Proinflammatory Effects of Heme on Immune and Non-Immune Cells

Heme is an endogenous danger-associated molecule that can induce proinflammatory programs in various immune and non-immune cells [112–115]. Exposure of endothelial cells to heme results in mobilization of WPB, expression of adhesion molecules, and secretion of chemokines [11,27,33]. Heme also activates neutrophils (promoting migration, generation of reactive oxygen species, and the release of neutrophil extracellular traps), and induces the secretion of proinflammatory cytokines from macrophages (shown in vitro). It can thus contribute to systemic inflammation [26,116–118]. The proinflammatory activity of heme can be explained in part by its ability to act as an agonist of TLR4, as shown in vitro in human and mouse macrophages, neutrophils, or endothelial cells, as well as in mouse models of sickle-cell disease [26–28,118]. In most of these studies, particular attention has been paid to avoid lipopolysaccharide (LPS) contamination of heme preparations, which could trigger TLR4 signaling. Moreover, LPS-free methemoglobin has also been shown to activate TLR4 on macrophages and microglial cell lines in vitro and in vivo when injected into the rat subarachnoid space in the brain (subarachnoid hemorrhage model) [119]. Heme can activate the inflammasome, as has been shown in mouse models of hemolysis as well as in vitro in mouse macrophages [28]. The substantial proinflammatory activity of heme has been implicated in the pathogenesis of various diseases. Thus, ‘free’ heme has been demonstrated to induce vaso-occlusion and acute lung injury in a mouse model of sickle-cell disease via activation of TLR4 [27,32]. Heme also amplifies hemolysis and exacerbates hemolytic pathologies [32]. In addition, animal models have suggested that extracellular heme might be responsible for the severe forms of malaria (mouse models of experimental cerebral malaria) and that it may play a role in the pathogenesis of subarachnoid hemorrhage (mouse model of prechiasmatic injection of autologous blood) [120] and of septic shock (mouse model of cecal ligation and puncture) [111,121,122]. Recent reviews have summarized the pathophysiological effects of heme in various disease contexts [7,14,28,92,103].
interactions. This ‘cell-free’ heme can be bioavailable for various biochemical reactions (described below). Interestingly, a recent study demonstrated that, in severe hemolytic disease (sickle cell anemia), at least one third of plasma heme was associated with membrane vesicle structures (microparticles) that were generated from erythrocytes during hemolysis [24]. As such, erythrocyte-derived microparticles might serve as a vehicle for heme delivery, and have been proposed to induce damage when transferring heme to human endothelial cells in vitro [24].

Most studies concerning the pathophysiological roles of heme have focused on the protective effect of the heme-degrading enzyme, heme oxygenase 1 (HO-1) [25] (Box 2), and on the effect of this danger-associated molecule on cells, leading to oxidative stress, TLR4 signaling [26,27], and NLRP3 inflammasome activation [28] (Box 4). However, during pathological conditions, heme released into plasma may also interact with, and affect the activity of, different plasma proteins, especially following exhaustion of hemoglobin/heme scavengers. Heme can then influence the functions of hemostatic proteins, the complement system, and circulating immunoglobulins. The interactions of heme with blood plasma effector systems may thus contribute to complex pathological mechanisms in hemolytic diseases. Increased understanding of these processes may lead to the development of novel therapeutic approaches for the treatment and/or management of hemolytic diseases. In this review we systematically analyze current knowledge on the functional repercussions of heme interactions with essential plasma system proteins, focusing specifically on hemostasis, complement, and antibody molecules (Box 5).

The Complex Relationships of Heme with Actors of Primary and Secondary Hemostasis

Hemolytic disorders are frequently associated with enhanced coagulation, thrombotic activity, and endothelial activation [29–31]. For example, one of the severe complications of sickle-cell disease is vaso-occlusion, an outcome that can be triggered in mice upon heme administration [27,32]. Heme is one of the factors capable of inducing stimulation and damage of endothelium, promoting the recruitment of neutrophils and sickle-cell erythrocytes, and subsequently prompting a vaso-occlusive crisis. This effect has been shown to be mediated in part by heme-mediated degranulation of Weibel–Palade bodies (WPB), as demonstrated in vitro using human endothelial cells (HUVEC). Ultra-large von Willebrand factor (UL-VWF) and the adhesion molecule P-selectin are secreted from endothelial cell WPBs [27,33,34] (Figure 1).
Box 5. Plasma Effector Systems

Blood Coagulation

Following vascular injury, endothelial cells, platelets, and coagulation proteins cooperate in a multistep process which involves different proteins, receptors, and signaling pathways with the goal of limiting blood loss. The coagulation cascade can be triggered by two pathways which lead to fibrin formation. The contact (intrinsic) pathway is triggered by the interaction of negatively charged cell surface with factor XII (FXII); its autoactivation generates activated FXIIa (FXIIa), FXIIa cleaves FV, generating FXa that subsequently cleaves FIX into FIXa. The latter, in association with FVIIIa, Ca²⁺ and phospholipids, generates FVa by cleaving FX. The extrinsic pathway is triggered by the exposure of tissue factor (TF) on endothelial cells; the subendothelium binds FVIIa, cleaving FX into Fxa. Fxa, in association with FVa, Ca²⁺, and phospholipids, cleaves prothrombin (FII) into FIIa (thrombin), which ultimately converts fibrinogen into fibrin. It also cleaves and activates FVIII and FV, amplifying the coagulation cascade. The activation of endothelial cells induces the expression of proadhesive and procoagulant molecules such as von Willebrand factor (VWF), P-selectin, ICAM-1, and VCAM-1, triggering the adhesion and activation of platelets and leukocytes as well as the expression of TF. Subsequently, these events lead to the activation of the coagulation cascade and to clot formation.

Immunoglobulins

Immunoglobulins are plasma glycoproteins produced by B lymphocytes with important roles in immune defense. The binding sites of immunoglobulins are characterized by enormous sequence and structural heterogeneity, allowing widespread recognition of molecular structures. Immunoglobulin G (IgG) is the second most abundant protein (after albumin) in plasma, with average concentrations of 74 μM in healthy individuals [83].

The Complement System

Complement is a part of the innate immune system which plays an essential role in defense against pathogens and in tissue homeostasis [123]. The complement system consists of a large number of blood proteins as well as membrane-associated regulators and receptors. Depending on the initiation mechanism, complement activation is divided into the classical, lectin, and alternative pathways. The classical pathway is activated through the interaction of the recognition component C1q with antigen-bound IgM or IgG. The lectin pathway of complement is activated following recognition of mannose-containing glycans by the mannan-binding lectin. The alternative pathway is permanently active owing to spontaneous activation and transformation of the bio-inactive component C3 to a bioactive C3b(H2O) form [81,123]. The three pathways converge to the central effector component C5b to execute a common terminal cascade of reactions. Under physiological conditions, activation of the complement system is tightly regulated. However, in distinct pathological situations, dysregulated complement activation occurs, leading to severe tissue damage and inflammation [86].

Indeed, free heme has a potent prothrombotic activity, mediated in part by the release of UL-VWF and P-selectin (Figure 1). This mediates adhesion of platelets to UL-VWF, and of leukocytes to the endothelium [35]. A recent study has also revealed that bilirubin, a metabolite of heme degradation, also present at elevated concentrations in patients with hemolytic diseases, binds to UL-VWF and prevents its enzymatic cleavage into VWF by the protein ADAMTS13 [36] (Figure 1). Impaired cleavage of UL-VWF results in increased prothrombotic activity at the endothelium. Moreover, in vitro experiments have demonstrated that heme binds to human platelets and induces platelet activation and aggregation [37–39]. These observations indicate that heme and its metabolite derivatives harbor prothrombotic activities which potentiate platelet activation and aggregation.

Heme has been used for the treatment of acute porphyria episodes [40]. Occasionally, transient perturbations in hemostatic functions, such as the prolongation of thrombin time (TT), activated partial thromboplastin time (aPTT) and prothrombin time (PT), were reported in heme-treated patients [38,41]. This was partially due to a decrease in the concentration and activity of the cofactors of the intrinsic coagulation pathway, factor V (FV) and factor VIII (FVIII) (Figure 1 and Box 5). In vitro experiments using radiolabeled heme revealed that heme interacts with purified human FVIII [37]. Further binding analyses revealed that this interaction occurs at high affinity (KD ≤0.1 nM), exceeding that of heme binding to albumin (KD ≤10 nM) [42]. Heme significantly reduced FVIII cofactor activity by inhibiting its interaction with activated factor IX (FIXa) in vitro. Under physiological conditions, VWF forms complexes with FVIII [43]. In humans

plays important roles in immune defense.

**Complement receptor 1 (CR1):** membrane-expressed protein that specifically binds to the C3 and C4 activation fragments.

**Decay-accelerating factor (DAF):** a protective and negative regulator of the complement system. DAF is expressed on the membrane surface of host cells.

**Factor H:** plasma protein negative regulator of the complement system. It regulates the alternative pathway C3 convertase.

**Fibrinogen:** abundant plasma glycoprotein that participates in the formation of blood clots, following enzymatic activation by thrombin, to form fibrin and stable clots.

**Factor V and factor VIII (FV, FVIII):** plasma glycoproteins proteins that play a cofactor role for FIXa and FVIIa respectively. Absence of functional FVIII results in hemophilia A.

**Haptoglobin:** plasma protein that binds to and scavenges hemoglobin.

**Heme:** iron protoporphyrin IX, a macrocyclic compound consisting of a tetrapyrrole ring and iron ion. Heme is used as a prosthetic group by numerous proteins for gas exchange and the catalysis of oxidation reactions.

**Hemolysis:** pathological process accompanied by loss of membrane integrity of red blood cells and release of their intracellular contents (predominantly hemoglobin).

**Heme oxygenase 1 (HO-1/hsp32):** inducible heme-degrading enzyme with potent cytoprotective and anti-inflammatory effects. HO-1 is upregulated as a result of cellular stress.

**Hemopexin:** plasma protein that binds to heme with very high affinity (KD <1 nM).

**Hemoproteins:** proteins that use heme as a prosthetic group or cofactor. Examples include hemoglobin, myoglobin, cytochrome c, catalase, peroxidase, etc.

**Membrane cofactor protein (MCP/CD46):** a protective negative regulator of the complement system. MCP is expressed on the membrane surface of host cells.

**Opsonization:** process of binding biologically active molecules (antibodies, complement) on particulate matter (viruses, bacteria, eukaryotic cells, cell debris etc.).

**Paroxysmal nocturnal hemoglobinuria:** a rare, acquired
and mice, VWF protects FVIII from degradation by proteases in the circulation and controls FVIII catabolism [43]. *In vitro* studies have shown that heme binding does not impair the interactions of human FVIII with VWF or phospholipids [42]. In addition, upon thrombin activation, heme binding to the FVIII–VWF complex prevented dissociation WW from FVIII [37]. Human WWF, however, protects human FVIII very efficiently from *in vitro* inactivation by heme [42].

From another perspective, *in vitro* assays have demonstrated that heme can also bind to fibrinogen and decrease its thrombin-mediated cleavage, thus affecting the final common coagulation pathway and reducing fibrin formation, important in clotting [44] (Figure 1). Importantly, fibrinogen copurifies in complex with heme in plasma from healthy individuals [44]. Heme perturbs the enzymatic activity of thrombin, further contributing to the overall anticoagulant effect and decreased fibrin formation [45]. Similar effects on coagulation have been reported after administration of heme to healthy volunteers [46]. Thus, exposure of coagulation proteins to heme, in particular FVIII, FX, FV, and fibrinogen, alter fibrin generation. This may explain, at least in part, the anticoagulant potential of heme on the intrinsic and terminal pathways of the coagulation cascade [45]. However, the anticoagulant activity of heme is transient and disappears after discontinuation of treatment. Later studies have suggested that the anticoagulant effect of heme might be ascribed to its degradation product(s) [47–49]. In contrast to the anticoagulant effect of heme on the intrinsic and terminal pathways of coagulation, heme can also trigger the extrinsic pathway of coagulation by upregulating tissue factor (TF) on the surface of human endothelial cells *in vitro*, as well as on leukocytes and perivascular cells in mice [50,51]. In the extrinsic pathway of coagulation, upregulated TF binds to activated factor VII (FVIIa), thus leading to activation of FX. Blocking TF with antibodies or via genetic ablation has been shown to reduce the procoagulant activity of ‘free’ heme in a mouse model of sickle cell disease [51]. Taken sequentially, it appears that the release of heme under hemolytic conditions initiates the extrinsic pathway of coagulation through the upregulation of TF on endothelial cells and leukocytes, but subsequently blocks the propagation of coagulation by inhibiting FVIII and FX, and by inhibiting the conversion of fibrinogen into fibrin and fibrin clots. One might speculate that the delicate balance between the anticoagulant and prothrombotic effects of heme depends on the availability of heme to interact with different proteins and cell-surface receptors. The overall effects of heme on hemostasis might well be potentiated by additional factors, such as ongoing inflammation. We hypothesize that generally heme triggers thrombosis, but limits its propagation. Further data might help to elucidate this issue.

**Immunoglobulins Are Natural and Non-Inert Scavengers of Free Heme**

Immunoglobulins are among the proteins that circulate at the highest concentration in plasma (Box 5). They are thus prone to functionally relevant interactions with free heme under hemolytic conditions. About a decade ago, McIntyre observed that the *in vitro* incubation of blood or plasma from healthy humans with medium containing heme had a profound effect on the antigen-binding properties of immunoglobulins [52]. Thus, incubation with heme resulted in the appearance of new binding specificities of antibodies towards phospholipids and various protein autoantigens [52–65]. Importantly, upon exposure to heme, elicited antibody titers in normal human plasma satisfied the criteria for positivity in standard diagnostic assays for disease-associated autoantibodies. More recent work has demonstrated that monoclonal antibodies from mice or humans exhibit antigen-binding polyreactivity (i.e., the ability to recognize multiple unrelated antigens) following exposure to heme *in vitro* [56–58]. Immunoglobulins belonging to distinct classes (IgG, IgA, IgM, and IgE) were found to acquire new antigen-binding specificities upon exposure to heme, suggesting that this phenomenon is dependent on the variable regions of these antibodies [53,55,59,60].

The fraction of antibodies that acquire novel binding specificities in the presence of heme represents 10–25% of the human immunoglobulin repertoire [61,62]. These antibodies exhibit stem cell disorder characterized by chronic hemolysis, bone marrow failure, and venous thromboembolism. Complement-mediated hemolysis occurs due to lack of expression of the complement regulators CD55 and CD59 on red blood cells. **Porphyria**: a group of rare congenital diseases caused by mutations in enzymes responsible for heme biosynthesis. Functional impairment of these enzymes results in the accumulation of large quantities of heme precursors that exert various toxic effects. **Prothrombin time (PT)**: a functional *in vitro* test to measure to the extrinsic pathway and the common pathway of coagulation in plasma. **Sickle-cell disease (sickle-cell anemia)**: a congenital hematologic disorder caused by homozygous mutations in hemoglobin. It is accompanied by morphological changes in red blood cells resulting in increased hemolysis. It is often associated with infection, severe pain, and vascular pathology. **TAK-242**: a specific low molecular weight inhibitor of TLR4 signaling that binds to the intracellular domain of the receptor. **β-Thalassemia**: inherited autosomal recessive blood disorder, characterized by reduced synthesis of hemoglobin. **Toll-like receptor 4 (TLR4)**: innate immune receptor expressed on many types of cells. It recognizes bacterial products and initiates potent inflammatory reactions. **Thrombin time (TT)**: a functional *in vitro* test to measure clot formation by measuring the time needed for the formation of a fibrin clot in plasma after the addition of thrombin. **von Willebrand Factor (VWF)**: plasma glycoprotein involved in primary hemostasis. VWF deficiency results in a bleeding disorder called von Willebrand disease. **Weibel–Palade bodies (WPB)**: endothelial cell-specific vesicular structures containing many biologically active molecules, in particular ultra-large VWF and P-selectin. WPB formation is dependent on the presence of VWF. Following endothelial activation, WPB release their contents extracellularly into the blood and the subendothelium.
Figure 1. Role of Heme in the Initiation, Progression, and Stability of Thrombus Formation (Coagulation). Under hemolytic conditions, free heme is released in the blood following red blood cell (RBC) lysis. The exposure of endothelial cells to free heme induces endothelial activation associated with the release of ultra-large von Willebrand factor (UL-VWF) and P-selectin from Weibel-Palade bodies (WPB). P-selectin induces leukocyte (white blood cell, WBC) recruitment through P-selectin–PSGL1 (P-selectin glycoprotein ligand–1) interactions. Heme induces platelet activation and aggregation through different pathways: (1) the release of UL-VWF promotes platelet activation and aggregation by interacting with platelet GPIb (glycoprotein Ib), (2) heme inhibits the cleavage of UL-VWF into VWF by its protease ADAMTS13; excess prothrombotic UL-VWF can spontaneously bind to platelets and induce platelet activation and aggregation, and (3) heme directly induces platelet activation and aggregation. In parallel to the release of WPB, free heme upregulates tissue factor (TF) on endothelial cells and WBC, triggering the extrinsic pathway of coagulation. (4) Paradoxically, heme can also inhibit the propagation of the coagulation cascade by inhibiting cofactors FVIII and FV, as well as thrombin-mediated fibrinogen cleavage and fibrin generation.

Physiologically relevant affinities for their newly recognized antigens, with equilibrium affinities ($K_D$) between $10^{-8}$ and $10^{-9}$ M [58,60,63]. It has been proposed that the pro-oxidative potential of heme plays a role in the alteration of the binding specificity of immunoglobulins [53,55,59]. This hypothesis is supported by the observation that other pro-oxidative substances, including reactive oxygen species and ferrous ions, are also capable of conferring novel binding specificities to antibodies [53,55,64,65]. Importantly, in vitro modification of antibody specificity has been associated with the binding of heme to immunoglobulin molecules [56,58]. Noteworthy, antibodies sensitive to heme possess significantly lower numbers of somatic mutations in genes encoding their variable regions [61,62]. Generally, less-mutated antibodies possess enhanced structural flexibility of their antigen-binding sites [66]. Therefore, such antibodies might exhibit increased propensity to accommodate heme, and consequently be capable of reorganizing their antigen-binding sites.

However, the biological consequences of altered antigen-binding properties in immunoglobulins by heme remain ill-defined. The relatively high affinity of binding acquired towards different autoantigens suggests that heme-sensitive antibodies may have a pathogenic potential. For example, the in vitro appearance of strong reactivity towards phospholipids of antibodies from normal human plasma following exposure to heme suggests that heme, when released in vivo, may perturb coagulation processes indirectly via its effects on antibodies. Indeed, anti-phospholipid antibodies harbor substantial prothrombotic activity [67]. Conversely, based on in vitro studies, McIntyre and colleagues hypothesized that phosphatidylycerine-reactive antibodies
induced by heme exposure might also play a beneficial role [55]. For instance, these antibodies might facilitate the clearance of damaged or dying cells by binding exposed phosphatidylserine on the cell membrane leaflet; increased cell debris clearance could contribute to reducing risks of inflammation and autoimmunity. In support of this hypothesis, in patients with Alzheimer’s disease (a chronic neuroinflammatory condition), the levels of heme-sensitive antibodies in cerebrospinal fluid and blood have been reported to be significantly lower than in healthy individuals [68–70]. Furthermore, administration of heme-treated immunoglobulins to mice does not seem to trigger any pathological consequences. On the contrary, these preparations appeared to be beneficial in the treatment of mice with streptozotocin-induced experimental autoimmune diabetes (as evidenced by improved glycemic responses and other parameters) [71]. This suggests that heme-sensitive antibodies might bear immune-modulatory potential.

One of our working hypotheses proposes that the heme-induced acquisition of antigen-binding specificities by circulating immunoglobulins provides an additional mechanism of diversification of immune repertoires. Thus, heme could be envisaged as a danger signal that promptly diversifies the antigen-binding specificity of circulating antibodies upon pathogen encounter, hemolysis or tissue injury. This post-translational diversification of immune specificities might play an important role in regulating inflammation, but may also represent a risk for deleterious autoimmune reactions (Figure 2). Further studies are warranted to understand the mechanisms and physiological significance of the effect of heme on antibody specificity. In addition, the contribution of this phenomenon to the pathophysiological mechanisms of some or many hemolytic diseases remains to be determined.

**Heme-Associated Imbalance of the Complement System**

Complement is a powerful innate immune defense mechanism, but it can also induce host tissue damage upon pathological overactivation (Box 5) (Figure 3A). Hemolytic diseases are often accompanied by dysregulation and overactivation of the complement system [72–74], which may be induced by free extracellular heme [33,75–77] (Figure 3B). C3a and C5a anaphylatoxins, as well as the soluble membrane attack complex (sC5b9), are generated by incubation of heme with human serum or blood in vitro, via the alternative complement pathway [33,77] (Box 5). Furthermore, the incubation of serum or whole blood with heme induces deposition of activation fragments (C3b, iC3b, C3d) of complement component 3 (C3) at the surface of erythrocytes [77]. The complement receptor 1 (CR1) is indispensable for this process and serves as a platform for the binding of C3 activation fragments. It has been reported that heme-induced complement activation and opsonization of red blood cells with C3 fragments may have important repercussions for the pathogenesis of malaria [77]. Indeed, the severe forms of malaria are associated with activation of the complement system [74]. Plasmodium-mediated lysis of a single infected erythrocyte in vivo was shown to result in the lysis of 8–10 uninfected cells [78,79]. The mechanism behind this erythrocyte loss is not well understood but may be related to reduced erythrocyte deformability, accelerated senescence, or to complement or antibody-mediated (anti-erythrocyte) erythrophagocytosis [79]. It is possible that heme-induced overactivation of the alternative complement pathway, and deposition of C3 fragments on erythrocyte membranes, participate in erythrophagocytosis of uninfected red blood cells in severe forms of malaria [77]. This scenario may resemble that of paroxysmal nocturnal hemoglobinuria patients, who can be treated with complement C5-blocking antibody eculizumab [80]; in this example, extravascular hemolysis has been shown to occur in a small number of these patients despite treatment. It has been suggested that this is due to erythrophagocytosis of red blood cells deficient in the C3d-opsonized complement regulators – CD55 and CD59 [81,82]. Future studies should aim to validate the in vivo relevance of complement imbalances due to heme. There is clearly an urgent need to obtain further mechanistic insight into the pathways and extent of heme-induced complement activation and erythrocyte destruction in various hemolytic diseases, including paroxysmal nocturnal hemoglobinuria, malaria, and others.
In vitro studies of the mechanisms underlying heme-mediated activation of the alternative complement pathway have indicated that heme binds to human C3 [33]. In this report, heme was found to preferentially bind to intact C3 and C3a, but not to the C3 degradation fragments C3b or C3d [33]. Molecular docking predicted the interaction of heme in close proximity to a thioester bond, known to be important for the activation of C3. Moreover, in vitro exposure of human C3 to heme resulted in enhanced generation of C3(H2O) and enhanced homophilic interactions [33]. These effects might contribute to the activation of the complement system in the fluid phase. Heme exposure of other proteins in the alternative pathway (factors B, D, and H) does not appear to have any impact on their functions, thus implying that heme activates the alternative complement pathway by affecting C3 directly [33]. The fact that C3 is the most abundant complement protein in plasma (ca 8 μM) is also in favor of the hypothesis that there is a direct effect of heme on C3 [83].

It has been shown that brief preincubation of human primary endothelial cells (HUVEC) and glomerular endothelial cells with heme, followed by the addition of normal human sera, results in
Figure 3. Heme-Mediated Modulation of the Complement System. (A) Overview of the complement cascade: (1) The classical and lectin pathways are triggered on a target surface (pathogens or stressed/dying host cells) after a recognition event. The alternative pathway is permanently basally active due to spontaneous activation of the central complement component C3. (2) The three pathways converge at the level of C3 cleavage and generation of C3b fragments, opsonizing target surfaces. (3) Two anaphylatoxins are generated during complement activation, C3a and C5a, that have potent proinflammatory activity. (4) The common terminal pathway proceeds until the formation of the membrane attack complex that is able to activate host cells and to lyse particular bacteria. (B) Heme modulates the activity of the complement system: (1) heme binds to C3 and enhances its spontaneous hydrolysis, thus inducing overactivation of the alternative pathway. (2) Heme can inhibit C1q binding to target molecules.
the activation of the alternative complement pathway on cellular surfaces and leads to the deposition of C3 fragments [33]. Deposition of C3 fragments on endothelial cells may be due to increased expression of P-selectin [33], serving as a platform for C3b binding, as suggested by experiments using transfected cells and endothelial cells in vitro, as well as in a mouse model of Shiga toxin infection [84,85]. Moreover, exposure of endothelial cells to heme results in diminished surface expression of the negative complement regulators decay-accelerating factor (DAF) and membrane cofactor protein (MCP) [33], and hence reduces protection against complement. The effect of heme on the endothelium can have important pathological consequences, especially in conditions of genetic abnormalities of complement components. Indeed, incubation of heme-exposed endothelial cells with sera from patients with atypical hemolytic uremic syndrome (aHUS), harboring mutations in factor H or C3, leads to a significantly higher deposition of C3 fragments on cells compared to healthy sera [33]. aHUS has incomplete penetrance in mutation carriers, and primary and secondary hits are needed to induce a disease flare [86]. While infection, pregnancy, or some drugs have been proposed as primary hits, there is no explanation for the fact that some mutation carriers do not develop the disease despite the presence of infections or multiple pregnancies. The assumption has therefore been that there is a threshold of tolerable endothelial stress (caused by a primary trigger), and that an additional stress (caused by the secondary hit) is necessary to break the balance and trigger disease flare. Based on in vitro experiments, it could be hypothesized that heme released during hemolysis serves as a secondary hit, increasing endothelial stress above a tolerable threshold, thus leading to severe thrombotic microangiopathy lesions and full-blown aHUS [33]. Complement activation has been detected in the plasma of patients with hemolytic diseases (without genetic or acquired abnormalities in complement proteins). These include patients with sickle-cell disease during painful crisis [73,87] and patients with malaria [74,88]. However, the exact role of hemaolysis and heme-derived products in these processes is not well understood.

In addition to C3, heme has also been shown to modulate the functions of complement component C1q. However, in contrast to its effect on C3, exposure of C1q to heme was demonstrated to inhibit its functions in vitro [89,90] (Figure 3B). Heme could suppress C1q binding to its main ligands, immunoglobulins and C-reactive protein (CRP), in a concentration-dependent manner. This effect resulted in an overall suppression of the activation of the classical complement pathway [89,90] (Box 5). The inhibitory effect of heme on the classical pathway is in agreement with in vitro functional assays showing that heme is capable of activating only the alternative pathway in human sera. The inhibitory effect of heme on the molecular recognition by C1q is caused by a perturbation in the electrostatic properties of the protein [90], known to play a decisive role in modulating target recognition.

Such differential effects of heme on various components of the complement system (Figure 3) are reminiscent of its effects on blood coagulation. The ability of extracellular heme to directly activate C3 and the alternative complement pathway may have serious pathological consequences, inducing non-specific tissue damage and inflammation. Blockade of the classical pathway at the level of C1q could compromise the effector functions of antibodies and/or severely impair the clearance of apoptotic cells, further aggravating inflammation. Because the alternative pathway represents an amplification loop of all complement pathways, and is responsible for up to 80% of the terminal complement pathway activity [81], the activation complement pathway in the blood. The C3b generated binds to red blood cells (covalently or via CR1, CD35) or to endothelial cells (covalently or via P-selectin, CD62P) and activates complement locally. C3b(H2O) can bind to the surface of heme-exposed endothelial cells as well. As a result, heme-induced complement activation may cause erythrocyte destruction and endothelial cell activation. (2) In contrast to its overactivating effects on the alternative pathway, heme inhibits the classical pathway. It binds to C1q, alters its electrostatic properties, and hampers the recognition of target molecules. This results in a reduction of classical pathway C3 convertase formation and C3b deposition.
of C3 triggered by heme may have an overall dominant effect over that of heme-mediated C1q inhibition.

**Therapeutic Perspectives**

To fully appreciate the pathophysiological role of heme on plasma effector systems, one should consider its effects at a ‘global scale’ rather than on isolated elements of the system. Heme may be implicated and contribute to the development of (i) thrombotic microangiopathy in patients with aHUS [33], (ii) vaso-occlusion in sickle-cell disease [18,24,27,32], (iii) vascular injury and kidney dysfunction in transfusion of old blood [16], (iv) atherosclerosis [92], or (v) erythrocyte loss in malaria [77].

Because ‘free’ heme is an endogenous danger signal with potent proinflammatory activity, heme scavenging may be a potent therapeutic strategy to prevent tissue damage, inflammation, and thrombosis. However, here again the dualistic nature of heme must be taken into account. Despite its damaging effects, heme induces the expression of HO-1, which degrades heme to anti-inflammatory, cytoprotective, and antioxidant products [25]. Numerous studies demonstrate that preconditioning animals to free heme (via injection) induces cell adaptation and confers resistance to further tissue injury, as reported in mouse-to-rat cardiac xenograft transplantation [93] and in renal damage from ischemia/reperfusion in rats and mice [94,95]. Furthermore, a clinical trial has documented improvement of reperfusion patterns after ischemia in healthy subjects [96]. A recent Phase IIb clinical trial showed that preconditioning using heme upregulated HO-1 in renal transplantation, launching further studies on clinical outcome [97]. In another clinical trial, however, injection of heme-arginate did not improve endothelial function or insulin sensitivity in patients with metabolic syndrome [98]. This contrasts with the encouraging results so far obtained in preclinical animal models [99,100].

In view of these collective results, an ideal treatment against heme overload should aim to block tissue damage and overactivation of plasma effector systems, while at the same time preserving the capacity of heme to induce HO-1. Endocytosis of the heme–hemopexin complex by hepatocytes and macrophages has been shown to induce upregulation of intracellular HO-1 [101,102]. During massive hemolysis such as in hemolytic anemia, hemopexin is consumed, and this results in heme overload.

Another therapeutic approach has been proposed based on experiments using mouse models of sickle-cell disease [27,32]. In these, the inhibition of TLR4-mediated heme-dependent signaling by specific TLR4 inhibitors such as TAK-242 has suggested that limiting hemolysis-associated thrombosis, vascular damage, and immune cell-mediated damage might be achieved. It is unknown, however, whether TLR4 inhibitors would prevent the consequences of action of heme on plasma systems.

So far, supplementation of plasma-derived or recombinant hemopexin has shown efficacy in mouse models of sickle-cell disease and β-thalassemia [18], as well as in severe sepsis [103] and focal cerebral ischemia [104]. However, studies assessing the ability of hemopexin to prevent heme-dependent dysregulation of plasma systems are still lacking. Positive results in this arena would represent an advance towards the preclinical development of hemopexin as a drug.

**Concluding Remarks**

Heme has pleiotropic effects on plasma effector systems. It has the potential to interfere with the activity of several plasma proteins and, depending on the molecular target, can induce antagonistic effects resulting in loss or gain of function [1–5]. This apparent dichotomy might be explained by the physicochemical nature of the molecule: heme manifests high binding

**Outstanding Questions**

What are the molecular mechanisms underlying the versatile effects of heme on various plasma proteins? Depending on a given plasma protein, heme induces either loss or gain of function. There is evidence that heme exerts its effects by direct binding to plasma proteins. A mechanistic explanation of the observed functional alterations in different cases would require comprehensive biophysical, structural, and mutagenesis analyses.

Which form(s) of heme is(are) responsible for perturbing plasma system functions? The iron ion of heme can attain different oxidation states (Fe²⁺, Fe³⁺), and, in particular cases, Fe⁴⁺; heme exists as a monomer but, in aqueous solutions, spontaneously forms dimers or larger aggregates. The nature of the molecular species of heme that affect plasma proteins is not known. In addition, it has not been established whether heme in vitro has the same molecular features as free heme released from hemoproteins in vivo. Functional assessment of the effects of different molecular forms of heme on coagulation, complement, and immunoglobulins should be performed.

What is the overall effect of heme on hemostasis? When studied in reductionist in vitro systems, heme exerts both prothrombotic and anticoagulant effects. The overall effect of heme on blood hemostasis in vivo however remains unclear. A better understanding of the effects of heme will require assessment of hemostatic functions in carefully selected animal models.

What is the overall effect of heme on complement, and what is its in vivo relevance? When studied in vitro, heme activated the alternative pathway but inhibited the classical pathway. To gain a deeper understanding of the capacity of hemolysis-derived products, particularly heme, to modulate complement in vivo, C1q, C3 activation fragments, and C5b-9 deposition in vessels or tissues should be investigated in mouse models of drug-induced hemolysis and in other models of hemolytic diseases. Whether these effects are specific to heme should be verified via hemopexin injection. The levels of the complement biomarkers (Ba, sC5b-9) in the plasma of patients with hemolytic diseases needs to be evaluated. This might suggest to what extent
promiscuity [105] and has the potential to induce conformational changes upon binding to its target proteins.

Hemolytic disorders, either congenital or following infection, concern millions of individuals worldwide and are associated with elevated morbidity and mortality. Understanding the functional consequences of the interactions between hemolysis-derived heme and plasma proteins in effector systems may provide important knowledge on the roles of heme in the pathophysiology of hemolytic diseases (see Outstanding Questions). Presently, a better elucidation of the molecular mechanisms at play should encourage the development of novel therapeutic tools and strategies, facilitating the management of diseases associated with intravascular hemolysis.

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complement blockers (such as the monoclonal antibody eculizumab) could be used to manage or treat particular hemolytic diseases.

What are the molecular mechanisms underlying heme-induced changes in the antigen-binding specificities of antibodies? While some antibodies were suggested to use heme as a cofactor to extend their binding potential, direct structural evidence for this hypothesis is missing. Furthermore, the properties of the variable regions of antibodies that predetermine sensitivity to heme remain unknown. Molecular delineation of these parameters will rely on the resolution of the 3D structure of heme/immunoglobulin complexes and on systemic analyses of the repertoires of human monoclonal antibodies.

Does heme-mediated diversification of immunoglobulin antigen-binding specificities contribute to immune regulation? Such diversity is generated by genetic recombination and somatic mutations. The generation of appropriate B cell receptor transgenic mice might facilitate addressing whether heme-induced post-translational modifications altering the antigenic specificity of immunoglobulins contributes in any way to the generation of antibody diversity.

How do heme-mediated functional alterations of plasma effector systems (hemostasis, immunoglobulins, and complement) contribute to the pathogenesis of hemolytic diseases?

Could targeted blockade of heme-mediated pathways in plasma systems have therapeutic relevance? The effects of heme on plasma proteins are pleiotropic, and may perturb homeostasis under uncontrolled hemo- lytic conditions. Administration of exogenous heme scavengers (e.g., hemoxygen) may represent a therapeutic strategy to reduce the pathogenic effects of heme under hemolytic conditions and preserve the integrity of plasma effector systems.
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