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Hemoglobin and Heme Scavenger Receptors

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Abstract

Heme, the functional group of hemoglobin, myoglobin, and other hemoproteins, is a highly toxic substance when it appears in the extracellular milieu. To circumvent potential harmful effects of heme from hemoproteins released during physiological or pathological cell damage (such as hemolysis and rhabdomyolysis), specific high capacity scavenging systems have evolved in the mammalian organism. Two major systems, which essentially function in a similar way by means of a circulating latent plasma carrier protein that upon ligand binding is recognized by a receptor, are represented by a) the hemoglobin-binding *haptoglobin* and the receptor *CD163,* and b) the heme-binding *hemopexin* and the receptor *low density lipoprotein receptor-related protein/CD91.* Apart from the disclosure of the molecular basis for these important heme scavenging systems by identifying the functional link between the carrier proteins and the respective receptors, research over the last decade has shown how these systems, and the metabolic pathways they represent, closely relate to inflammation and other biological events. *Antioxid Redox Signal.* 12, 261–273.

Introduction

Intravascular hemolysis which usually accounts for ~10–20% of total red blood cell destruction (26) releases hemoglobin (Hb) from erythrocytes and their precursors into plasma. Outside the constraints of the red blood cell, Hb constitutes a potentially toxic compound. This toxicity stems from the ability of the heme group to engage in chemical reactions that result in the generation of free radicals (Fenton chemistry) which in turn may cause severe oxidative damage to tissues, in particular the kidney (57, 99). Another potentially toxic property of 'free' Hb is its ability to react with nitric oxide (NO), a signal molecule that plays a critical role in the regulation of smooth muscle relaxation, endothelial adhesion molecule expression, and platelet activation and aggregation (95, 98). Such Hb-mediated scavenging of NO reduces the bioavailability of NO and thus impedes NO homeostasis (95, 98).

Several molecular pathways that ensure rapid removal of 'free' Hb and heme have evolved to prevent the deleterious toxic effects associated with these substances. However, in special circumstances, such as in the case of infections, trauma, sickle cell anemia, and thalassemia, intravascular hemolysis may accelerate dramatically, leading to saturation of Hb/heme scavenging mechanisms. This results in a pathological state that is characterized by heme-mediated oxidative damage and a number of sequelae associated with limited NO availability, including smooth muscle dystonias, endothelial dysfunction, and thrombosis (95, 98). Patients in chemotherapy that affects monocytes/macrophages (such as CD33-targeted therapy in AML) are probably at highly increased risk of developing symptoms from 'free' Hb during intravascular hemolysis (Macrophage Depletion Syndrome) (63). In the present review we focus on the receptor-mediated scavenger pathways involved in detoxification of Hb and heme.

Scavenging of 'Free' Hemoglobin

The primary mechanism protecting against the deleterious effects of 'free' Hb is governed by a cooperation between the plasma protein haptoglobin (Hp) and the monocyte/macrophage scavenger receptor CD163 (49).

Haptoglobin

Hp is an abundant acute phase glycoprotein belonging to the α_2-globulin fraction of plasma (0.45–3 mg/ml plasma). It is composed of α- and β-subunits, representing a complement control protein domain and a serine protease domain, respectively (4, 31, 50, 51, 134, 140). Hp is above all expressed in the liver, although expression has also been detected to a lesser extent in lung, kidney, spleen, thymus, and heart (45, 139). The Hp plasma concentration rises during the acute phase response as a result of an increased rate of synthesis in response to inflammatory cytokines (65, 77, 84, 94). In contrast, levels are low or even undetectable during hemolytic

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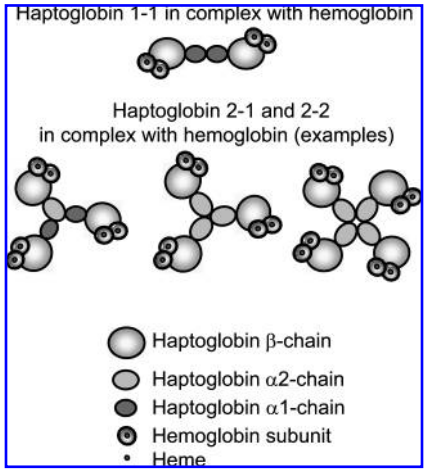

FIG. 1. Schematic representation of Hp–Hb complexes. The Hp1-1 molecule resembles a barbell-like elongated structure with two spherical head groups (the β-chains) connected by a thin filament with a central knob (the α-chains). The ($\alpha\beta$)-units of Hp1-1 are connected via inter-α-chain disulfide bonds mediated by cysteine 33. The Hb dimer binds the Hp1-1 β-chain off the axis in a *trans* configuration. Due to an intragenic duplication, the Hp α2-chain harbors two cysteines with the ability to mediate inter-α-chain disulfide bonds. Consequently, the Hp2-1 and Hp2-2 phenotypes display a complicated spectrum of Hp ($\alpha\beta$)-multimers. Examples shown here are the Hp2-1 trimer, the Hp2-2 trimer, and the Hp2-2 tetramer, all complexed with Hb. The illustrations are based on electron microscopy studies by Wejman *et al.* (131, 132).

episodes owing to an increased rate of endocytosis and subsequent lysosomal degradation (49).

In Hp, an α-subunit is linked to a β-subunit by means of disulfide binding. In its simplest form (corresponding to the version found in most mammals), Hp is composed of two such ($\alpha\beta$)-units connected by a disulfide bond between cysteine 33 of the two α-chains, thus forming an ($\alpha\beta$)-dimer [$(\alpha\beta)_2$] of $\sim$90 kDa (50, 131). Humans, however, harbor three Hp genotypes due to the presence of two different Hp alleles, hp1 and hp2, hence giving rise to the phenotypes Hp1-1, Hp2-1, and Hp2-2 (7, 61, 62, 112, 113, 140). Hp1-1, representing the aforementioned simplest form of Hp, is a barbell-like elongated structure with two spherical head groups (the β-chains) connected by a thin filament with a central knob (the α-chains), as described by electron microscopy (131) (Fig. 1). Being a result of a partial intragenic duplication of the hp1 gene, the hp2 allele encodes a slightly longer α-chain but is otherwise identical to the hp1 allele (62, 112). The duplicated region of the Hp2 α-chain comprises the cysteine involved in inter-α-chain disulfide bonding, and as a consequence, the Hp2-1 and Hp2-2 phenotypes display a pattern of various ($\alpha\beta$)-multimers (132) (Fig. 1).

The Hp α-chain appears primarily to serve to connect individual ($\alpha\beta$)-units to form dimers (Hp1-1 and Hp2-2 phenotypes) and multimers (Hp2-1 and Hp2-2 phenotypes) (49, 83). In contrast, the β-chain mediates interaction with binding partners such as Hb and CD163 (59, 83, 109, 114, 124, 125, 131, 132). Compared to the α-chain, the β-chain is generally more conserved between species (13). At the structural level, there are two evident differences between the Hp β-chain and serine proteases. Perhaps most obviously, the Hp β-chain lacks the obligatory catalytic triad of genuine serine proteases (50). In addition, one of the surface-exposed loops (referred to as loop 1) of the Hp β-chain is considerably enlarged compared to the corresponding loop in genuine serine proteases (31, 50, 89) (Fig. 2).

The binding between Hp and Hb is among the strongest noncovalent interactions reported in plasma (43). Once released into the circulation, Hb disintegrates into $\alpha\beta$-dimers that interact with Hp in a 1:1 stoichiometry [*i.e.*, one Hb dimer per Hp ($\alpha\beta$)-unit] (1, 18, 52, 79, 88, 109). Electron microscopy studies of the Hp(1-1)–Hb complex have revealed that one Hb dimer binds each Hp β-chain off the barbell axis in a *trans* configuration (131) (Fig. 1). A small region necessary for Hb binding has subsequently been mapped to residues 243–258 within the Hp1 β-chain (68, 83). Furthermore, other studies have disclosed that Hp1 β-chain residues 111–112, 230–233, and 238–239 may also be involved in Hb binding (59).

CD163: The Haptoglobin–Hemoglobin Receptor

Human CD163 (also known as M130, RM3/1 antigen, p155, or Hb scavenger receptor) is a 130 kDa glycoprotein almost exclusively expressed in cells of the monocyte lineage, with the highest expression detected in mature tissue macrophages, including Kupffer cells and red pulp macrophages (92, 126, 141).

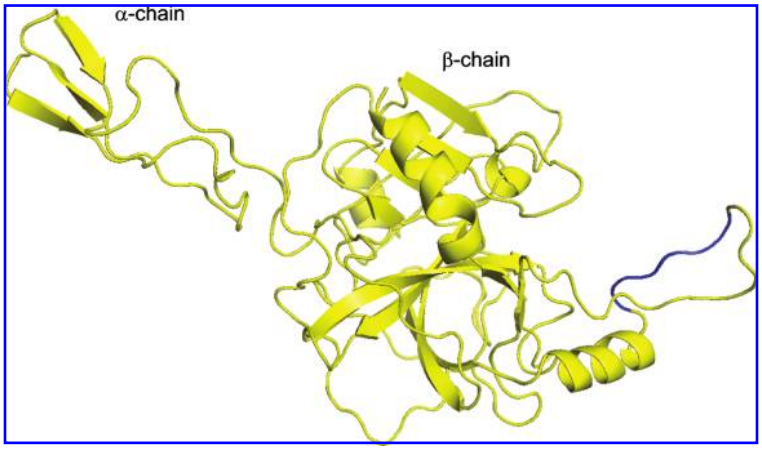

FIG. 2. Computer-modeled three-dimensional structure of the Hp1 ($\alpha\beta$)-unit. Prediction of the three-dimensional structure of the Hp1 ($\alpha\beta$)-unit is based on its homology with the complement components C1r and C1s (83). The residues of loop 1, thought to participate in CD163 binding, are highlighted in *blue*. Reprinted by permission from Nielsen *et al.* (83).

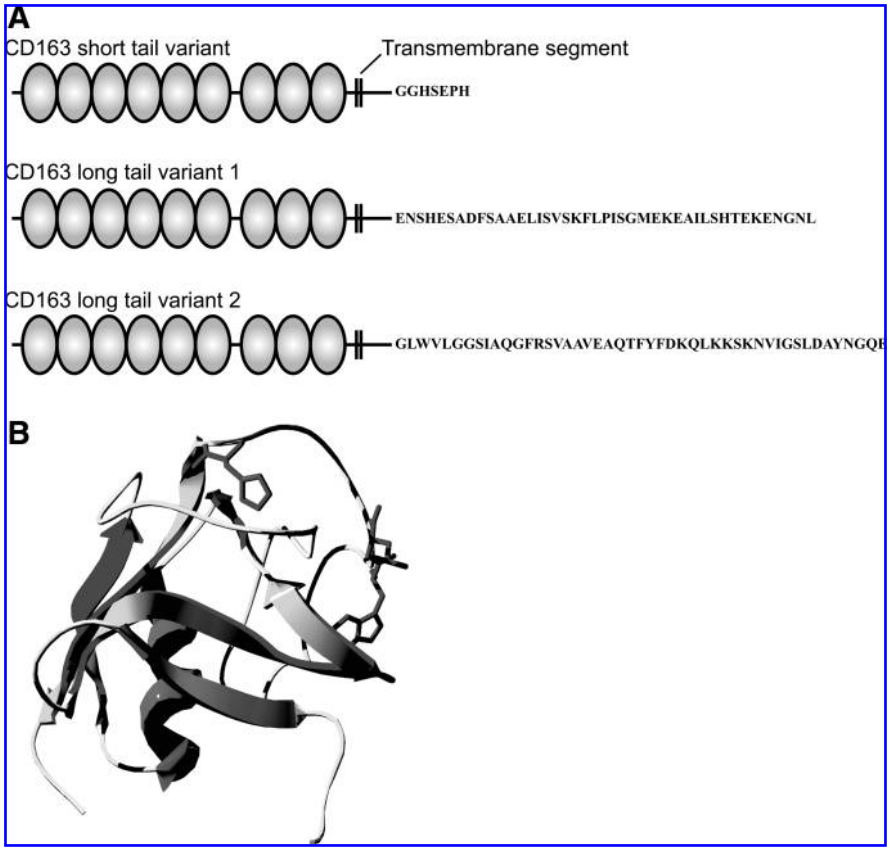

FIG. 3. Schematic representation of CD163 cytoplasmic tail variants and structure of the SRCR domain. (**A**) SRCR domains are indicated by *oval symbols*. The three variants have the extracellular region, the transmembrane segment, and the first 42 residues of the intracellular tail in common. The sequences of the divergent C-terminal tails are displayed. (**B**) Three-dimensional structure of the SRCR domain of the Mac-2 binding protein. The structure represents a six-stranded β-sheet cradling an α-helix. Shown in 'ball and stick' are the residues aligning with the ligand-binding residues of the CD6 SRCR domain 3. **B** is reprinted by permission from Graversen *et al.* (30).

CD163 is a member of the family of so-called scavenger receptor cysteine-rich (SRCR) domain-containing proteins. In addition to a 24-residue transmembrane segment and a short carboxy-terminal cytoplasmic tail, CD163 harbours a large extracellular region comprising nine SRCR domains (numbered 1–9 from the amino-terminus), only interrupted by a 31 residue interdomain segment between SRCR domains 6 and 7 (53) (Fig. 3).

The SRCR domain is an ancient and highly conserved cysteine-rich $\sim$100–110 residue domain present in a number of membrane-anchored and extracellular proteins typically playing a role in the immune system. The domain features a six-stranded β-sheet cradling a α-helix, as revealed by the crystal structure of the single SRCR domain of the Mac-2-binding protein (41) (Fig. 3). The SRCR domain is thought to mediate binding of ligands. Site-directed mutagenesis analysis of the membrane proximal SRCR domain of the CD6 protein suggests that the loop connecting β-strands 5 and 6 is involved in ligand binding (10, 110). As this particular region of SRCR domains is subject to a high degree of variation (10, 41), it is tempting to speculate that this loop determines the ligand specificity of individual SRCR domains.

CD163 displays particularly high homology to the SRCR-containing proteins CD5, CD6, M160 (also known as CD163B due to the high similarity to CD163), and WC1 (2, 32, 44, 136), harboring 3, 3, 12, and 11 SRCR domains, respectively. Interestingly, the 31 residue interdomain segment between SRCR domains 6 and 7 of CD163 is an integral component of a so-called 'long range cassette' also contained within M160 and WC1 (32, 53, 136) (Fig. 4). The functional role of this higher-order repeat remains unknown.

Several different CD163 splice variants have been reported, including three with identical extracellular and transmembrane regions, but different cytoplasmic regions (53, 97). These CD163 cytoplasmic tail variants are denoted the CD163 short tail variant (also known as the CD163 abundant or predominant form (53, 80)) and the CD163 long tail variants 1 and 2 (also known as the intracellular variants AC1 and AC2). The three variants have a common membrane proximal intracellular region of 42 amino acids, but their extreme carboxy-termini differ with respect to both sequence and

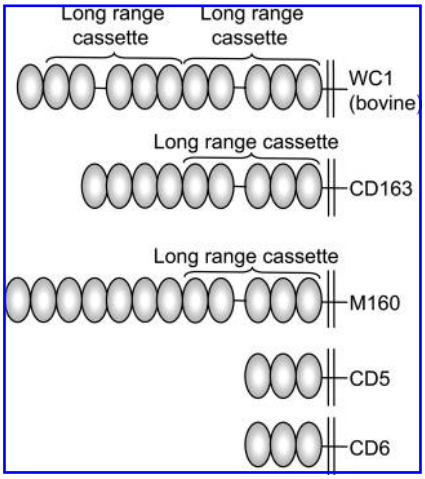

FIG. 4. Schematic representation of CD163 family members. The so-called 'long range cassette' present in CD163, WC1, and M160 is indicated.

length (53). Whereas the CD163 short tail variant has a cytoplasmic tail of 49 amino acids, the CD163 long tail variants 1 and 2 have cytoplasmic tails of 84 and 89 residues, respectively (Fig. 3).

The common membrane proximal region of the CD163 intracellular variants comprises a YXXφ-based endocytic motif (φ represents a bulky hydrophobic amino acid) (11), as well as candidate motifs for phosphorylation by protein kinase C and casein kinase II (96). The tail sequence unique to the CD163 long tail variant 1 harbours additional potential phosphorylation sites (96).

An extracellular variant of CD163 containing a 33 amino acid insertion between SRCR domains 5 and 6 has also been described (38, 53). Apart from this insertion, this variant is identical to the CD163 short tail variant. The short tail variant is the predominant CD163 mRNA species produced in human monocytes and the CD163-expressing SU-HDL cell line whereas the extracellular variant displays the lowest expression level (38, 53, 80).

CD163 mRNA and protein are induced by glucocorticoids (14, 38, 39, 76, 103, 117, 126, 133, 141). In addition, CD163 is upregulated by the acute phase mediator interleukin-6 (IL-6) and the anti-inflammatory cytokine IL-10 (14, 117). Among 19 identified IL-10-inducible genes in human monocytes, CD163 in fact displayed the strongest upregulation (137). Negative modulators of CD163 expression include the pro-inflammatory mediators tumor necrosis factor-α, interferon-γ, and lipopolysaccharide (LPS) (14, 141).

In addition to the CD163 variant forms resulting from alternative splicing, a soluble version of CD163 (sCD163), thought to comprise all nine SRCR domains, circulates in human plasma ($\sim$2 mg/L) (75, 116). This soluble version of CD163 arises from proteolytic shedding of the membrane-bound version by means of an as yet unknown metalloprotease (36, 66). The shedding is reportedly induced by phorbol 12-myristate 13-acetate, cross-linking of the Fcγ receptor in a protein kinase C-dependent manner, LPS through Toll-like receptor (TLR) 4 activation, as well as by ligands activating TLR2 and TLR5 (Fig. 5) (20, 36, 118, 129, 130). This indicates that CD163 is shedded from the cell surface as a result of the macrophage activation elicited by extracellular pathogen infections.

No definite functions have yet been established for sCD163. It is possible that sCD163 may aid removing Hp–Hb complexes (discussed below). The acute phase behavior with high concentrations present a few hours after LPS administration *in vivo* (36) may indicate some functional role in the innate immune response [*e.g.*, acting as an opsonin since CD163 may bind bacteria (22)]. Inhibitory effects of sCD163 on lymphocytes have also been described (24, 40).

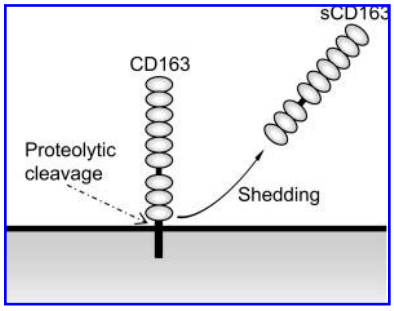

FIG. 5. Shedding of CD163 from the plasma membrane. CD163 is cleaved by means of an as yet unknown metalloprotease to form sCD163. Shedding is induced by phorbol 12-myristate 13-acetate, LPS, and cross-linking of the Fcγ receptor.

Due to the highly specific expression of CD163 in monocytes/macrophages, Schaer *et al.* (106) investigated whether sCD163 may function as a disease marker in conditions with macrophage activation and proliferation. In patients with hemophagocytic syndrome/macrophage activation syndrome, sCD163 is highly elevated (Table 1) and the levels closely follow the clinical course of the patients (106). The syndrome is characterized by overt hemophagocytosis by activated, morphologically benign macrophages, leading to life-threatening cytopenias. It is caused by severe hypercytokinemia as the consequence of a highly stimulated but ineffective immune response. Since diagnosis is often delayed due to the unspecific sepsis- or DIC-like symptoms, sCD163 may be a valuable tool to facilitate prompt medical therapy (21). Also, in critically ill patients with severe sepsis or severe liver disease, sCD163 is highly elevated, and several studies have consistently found a relation between increased sCD163 and poor outcome (25, 37, 48, 73, 74). It has been proposed that increased levels in sepsis and liver failure may reflect unrecognized hemophagocytosis (73, 105). Also, patients with increased macrophage-load due to inherited glucocerebrosidase deficiency (Gaucher disease) have highly increased sCD163 (Table 1) reflecting the accumulation of macrophages in bone marrow, spleen, and other tissues (72).

Macrophages are important players of inflammatory and autoimmune diseases, and CD163 is, for example, strongly

Table 1. sCD163 in Selected Clinical Conditions

	sCD163 (mg/l)			*Putative clinical use*		
	Mean	*Range*		*Diagnosis*	*Prognosis*	*Monitoring*
Normal subjects	2	1	4			
Hemophagocytic syndrome	38	20	160	+++	+++	+++
Sepsis	7	1	32		+++	?
Acute liver failure	22	4	76		+++	?
Tuberculosis	3	1	30		+++	?
Gaucher disease	9	4	24	+	?	++
Celiac disease	Increased				?	+++

expressed in inflamed synovium in rheumatoid arthritis and in microglia of multiple sclerosis patients. It is possible that sCD163 in some of these conditions may be of value as a specific marker to be used in combination with more unspecific markers of the acute phase response, such as CRP. Promising results have been obtained in systemic juvenile arthritis (which often has a component of hemophagocytosis) (9) and in celiac disease (19).

Scavenging of Hemoglobin by Haptoglobin and CD163

When released into plasma, Hb is instantly captured by Hp to form a complex of very high affinity (43). Binding of Hp to Hb has an inhibitory effect on the Hb toxic properties and prevents peroxidative modification of Hb (15). Moreover, the complex formation with Hp impairs filtration of the relatively small Hb molecule by the kidney (23, 47) and, instead, facilitates delivery of Hb to monocytes/macrophages expressing CD163. The latter binds and conveys cellular uptake of the Hp–Hb complex by receptor-mediated endocytosis (49). Inside the cell, the globin moieties are degraded in the lysosome [thus explaining the low or even undetectable levels of Hp in serum of patients with extensive hemolysis (16)]. The liberated heme is converted into less toxic compounds by heme oxygenase-1 (HO-1) in the cytosol (5). This Hb scavenging mechanism thus effectively protects against heme-mediated oxidative damage in the vascular system, as well as in the kidney (6, 57, 58), and may impair NO scavenging by Hb. The Hp-CD163-dependent clearance system is thought to function principally in liver and spleen macrophages, in addition to peripheral blood monocytes (101) that have the advantage of being able to act immediately at the site of red blood cell destruction, thereby limiting the physiological side effects of 'free' Hb.

CD163 mediates Hp–Hb internalization by means of the YXXφ-based endocytic motif situated in the membrane-proximal region common to all three cytoplasmic tail variants (80). Among the three variants, the short tail variant displays the highest endocytic efficacy, probably due to its much more pronounced degree of cell surface expression compared to the long tail variants that are mostly located in intracellular compartments (trans Golgi network/endosomes) (80). Upon internalization and delivery of its cargo to early endosomes, CD163 recycles to the plasma membrane ready to initiate another round of endocytosis (80, 100, 101). Interestingly, the heme carrier protein 1 (also known as proton-coupled folate transporter/heme carrier protein 1 (PCFT/HCP1)), reported to be involved in low affinity heme absorption in the duodenum, is expressed in human macrophages (93, 102, 108). Instigated by this observation, Schaer *et al.* recently performed a series of colocalization studies (102). These studies disclosed a striking spatial convergence of CD163-internalized Hp–Hb and PCFT/HCP1, as well as the two major splice variants of the nonheme iron transporter DMT1, DMT1-A and DMT1-B. Based on these data, Schaer *et al.* propose that Hp–Hb, upon internalization by CD163, first passes through PCFT/HCP1- and DMT1-B-positive early endosomes. The heme released from Hb at this point is speculated to be exported by PCFT/HCP1 to the cytosol whereas DMT1B is suggested to export any iron liberated by HO-1-independent activity. Remaining Hb, as well as heme-free globin chains, then traffic to DMT1-A-containing late endosomes/lysosome, where globin is degraded and the released heme exported to the cytosol by an as yet unidentified transporter (102). By analogy with DMT1-B in the early endosomes, DMT1-A could account for export of heme-derived iron from the late endosomes/lysosome. Finally, the exported heme is degraded by HO-1 whose expression is induced by the heme moiety of the internalized Hp–Hb (100).

The high affinity interaction between Hp–Hb and CD163 requires Ca^{2+} (49, 60) and critically depends on SRCR domain 3 of CD163 and three residues (glutamate 261, lysine 262, and threonine 264) positioned in the Hp β-chain, as deduced by deletion analyses of CD163 and mutational analyses of Hp, respectively (60, 83). According to data from the latter study, the mentioned three residues of the Hp β-chain are positioned in the unique extension of loop 1 of the Hp serine protease domain and appear to be directly involved in receptor binding (Fig. 2). The Hb-binding site on the Hp β-chain is positioned immediately amino-terminally to this CD163-binding site (68, 83). CD163 binding of Hp–Hb is Hp phenotype-dependent as CD163 has been shown to display a higher functional affinity for Hp(2-2)–Hb complexes than for Hp (1-1)–Hb complexes (49), a finding that is probably explained by the multiple receptor binding sites present in oligomeric Hp–Hb complexes.

As opposed to the high affinity CD163 binding triggered by Hp–Hb complex formation (49), Hp alone appears completely unable to bind the receptor (49, 83, 104). Hb, however, exhibits low affinity binding to CD163 and is internalized by CD163 in the absence of Hp, as reported in a study by Schaer *et al.* (104). According to their data, Hp critically promotes CD163-mediated Hb clearance at low, but not at high ($\geq$100 μg/ml) concentrations of 'free' Hb, leading to the proposal of a concentration-dependent biphasic model for Hb clearance. In this model, low amounts of Hb are cleared by high affinity binding of Hp–Hb to, and uptake by, CD163, whereas Hp-independent uptake of Hb may occur in situations with pronounced hemolysis in which the amount of 'free' Hb exceeds the binding capacity of Hp. In the same study, it was furthermore observed that high concentrations of Hb compete with Hp–Hb for binding to CD163. This implies that the Hb low affinity binding site overlaps with the Hp–Hb high affinity binding site (104).

Several studies point to a connection between the Hp-CD163-HO-1 system for Hb clearance/metabolism and an anti-inflammatory response, an association that has been established due to the anti-inflammatory, antiapoptotic, antiproliferative, and antioxidant properties of one or more of the heme metabolites carbon monoxide, biliverdin (which is quickly converted to bilirubin by the biliverdin reductase), and Fe^{2+}(71, 85). Due to the beneficial effect of these metabolites, HO-1 is often regarded as possessing a protective function. In this respect it is interesting to note that therapeutic molecules such as IL-10 and prostaglandin J_2 in fact work through their ability to activate HO-1. Owing to this, it has been proposed that HO-1 functions as a so-called 'therapeutic funnel' conveying the favorable effects of these therapeutic molecules (85). Since Hp, CD163, and HO-1 are all upregulated by the inflammatory cytokine IL-6 (69) it seems reasonable to speculate that these proteins may be simultaneously induced during inflammatory conditions in order to improve the rate of Hb clearance and metabolism.

It has been proposed that protein phosphorylation-dependent intracellular signalling cascades initiated by binding of Hp–Hb to CD163 on the cell surface may also contribute to this anti-inflammatory response (33, 90, 96, 115, 126). At any rate, cross-linking of CD163 at the cell surface by means of antibodies was reported to induce intracellular signal transduction leading to mobilization of Ca^{2+}, synthesis of inositol triphosphate, and secretion of IL-6 and granulocyte-macrophage colony-stimulating factor (126). Likewise, Hp–Hb binding to CD163 has been suggested to trigger signal transduction events, resulting in the secretion of IL-6 and IL-10 (33, 90). The secretion of IL-10 was reported to result in induction of HO-1 (90). With the upregulatory effect of IL-6 on Hp, CD163, and HO-1 in mind (69), in addition to the reported upregulatory effect of IL-10 on CD163 and HO-1 (14, 54, 117, 137), it is tempting to envisage IL-6 and IL-10 as part of positive feedback systems increasing the capacity for Hb clearance and metabolism.

A recent study reports the presence of a novel macrophage phenotype in hemorrhaged atherosclerotic plaques (12). This particular macrophage population is defined by high levels of CD163, but low levels of human leukocyte antigen-DR. Notably, the authors were able to reproduce the very same phenotype by exposing monocytes to Hp–Hb. However, when including antagonistic anti-CD163 antibodies, the differentiation process was blocked. In agreement with the high levels of CD163, the novel macrophage phenotype shows an increased capacity for Hb clearance and is among other things, characterized by less hydrogen peroxide release and reduced oxidant stress, in addition to increased cell survival. It thus seems reasonable to speculate that the release of Hb due to intraplaque hemorrhage directs monocyte/macrophage differentiation towards an antioxidant and athero-protective phenotype through a pathway involving Hp–Hb binding to CD163. Interestingly, and in agreement with the mentioned possible existence of positive feedback loops, antibodies directed against IL-10 inhibited this differentiation process (12).

While the models for CD163-mediated signaling and positive feedback systems appear very attractive, experiments performed by Schaer and colleagues talk against the existence of signal pathways and IL-10 secretion elicited by Hp–Hb binding to CD163 (100). According to their studies, CD163 functions only to transport Hb and the internalized heme is responsible for the upregulation of HO-1 (100). They propose that endotoxin contamination of commercially available Hb may have given rise to the secretion of IL-10 that was reported by others.

In future studies it will be pertinent to establish the physiological relevance of signal transduction initiated by Hp–Hb interaction with cell surface CD163. If CD163-mediated signaling proofs to be a physiological phenomenon, alternative splicing of the intracellular tail may be speculated to affect the extent of CD163 phosphorylation and/or signaling properties (96, 115). In addition, the signaling cascades could vary according to the nature of the particular ligand bound by CD163. For instance, it has been reported that Hp(1-1)–Hb binding to CD163 stimulates secretion of IL-6 and IL-10 to a much greater extent than does Hp(2-2)–Hb (33). In this way several different signal pathways could be envisaged, depending on the particular combination of Hp phenotype and CD163 variant involved.

Other Fates of 'Free' Hemoglobin

While it is generally accepted that the majority of 'free' Hb is cleared by the CD163-dependent pathway, other systems for Hb clearance and/or detoxification may exist. sCD163 is a low avidity binder of Hp–Hb [(60) and unpublished results] and could thus be speculated to contribute in some way to Hb neutralization/detoxification.

Another plasma protein, the Hp-homologous protein Hp-related protein (Hpr), was recently disclosed as an Hb binding protein (82). Hpr is a constituent of a minor subfraction of high density lipoprotein particles called Trypanosome Lytic Factor 1 (TLF1) displaying innate immunity against certain trypanosome parasites [reviewed by Nielsen *et al.* (81)]. Subsequent to endocytosis by the bloodstream-localized trypanosome, TLF1 elicits lysis of the parasite. The discovery that Hpr forms high affinity complexes with Hb (82), thereby enabling Hb to associate with TLF1 (82, 135) led to a recent breakthrough in elucidating the mechanism underlying the innate immunity function of TLF1 (127). In fact, the Hpr–Hb complex within TLF1 has recently been shown to be required for the trypanosome killing activity of TLF1 by serving as the ligand for an endocytic receptor on the surface of the trypanosome (127).

Apart from this critical function, a possible role for Hpr/TLF1-associated Hb remains currently unclear. Owing to the considerably higher concentration of Hp in normal human serum compared to Hpr [$\sim 40\ \mu g/ml$ in normal human serum (78, 82)], Hb is not expected to bind Hpr under normal circumstances. Nevertheless, in the case of massive hemolysis leading to deprivation of Hp from plasma, Hpr would be able to bind Hb in plasma. Strikingly and in contrast to Hp-bound Hb, Hpr–Hb does not bind CD163 as the three critical Hp residues located in the CD163 recognition loop of Hp are replaced in Hpr (82, 83). Thus, Hpr/TLF-bound Hb probably escapes CD163-mediated scavenging. Moreover, Hpr levels appear, unlike Hp levels, to be unaffected by intravascular hemolysis (78, 82). Association of Hb with Hpr thus seems to prolong the plasma survival time of Hb. It remains to be determined whether the Hpr/TLF1-bound Hb retains its toxicity, although it has been speculated that the antioxidant apolipoprotein A-I (8, 27) of TLF1 may protect the circulatory system from Hb-mediated oxidative damage (46, 82). However, as a novel study by Watanabe *et al.* reports that association of HDL with Hp–Hb can convert HDL into pro-inflammatory particles (128), it could be interesting to determine whether the Hpr-mediated association of Hb with TLF1 regulates the inflammatory properties of this particular HDL-subspecies in a similar manner.

When the Hp–CD163-dependent pathway and other possible Hb scavenging systems are saturated as occurs during chronic hemolysis, the multi-ligand receptors cubilin and megalin expressed in the apical membrane of proximal tubules are thought to mediate renal reabsorption of Hb from glomerular filtrate (29). These receptors moreover have the ability to mediate renal uptake of the hemoprotein myoglobin (28).

Scavenging of 'Free' Heme

'Free' heme originating from the dissolution of Hb or other heme-binding proteins leaking into plasma is bound by the protein hemopexin (Hx, 0.4–1.5 g/l) with very high affinity (K_d of $<10^{-12}$ *M*) (120). This complex of Hx and heme is

subsequently cleared by receptor-mediated endocytosis, primarily by cells of the liver. The low density lipoprotein receptor-related protein (LRP, also known as CD91 or α_2-macroglobulin receptor) has been shown to conduct this internalization of Hx-heme (42).

Hemopexin

Hx is a 439 amino acid residue plasma glycoprotein that is mainly synthesized in the liver, and to a lesser extent in neurons of the central nervous system, peripheral nerves, and the retina (17, 119, 121). It constitutes two homologous ~200 residue domains (known as Hx domains), separated by a 20 residue linker region. The Hx domain presents as a disk-like structure composed of a four-bladed β-propeller fold (86). The two disk-like domains of Hx are oriented perpendicular to one another, and heme has been shown to bind between these two propeller domains in a pocket bounded by the linker region (86). Interestingly, the Hx domain is not confined to Hx and heme-related functions. In fact, at least 544 different viral, prokaryotic, and eukaryotic proteins are thought to feature Hx-like motifs (91), and the Hx domain is involved in rather divergent processes, such as activation/inhibition of matrix metalloproteinases, dimerization, and ligand/substrate binding (91).

Low Density Lipoprotein Receptor-Related Protein/CD91—The Hemopexin-Heme Receptor

The 600 kDa LRP/CD91 belongs to the low density lipoprotein (LDL) receptor superfamily and is involved in various endocytic and signal transduction pathways (35, 67). It recognizes >40 different ligands such as lipoproteins, extracellular matrix proteins, viruses, cytokines, and complexes between proteases and protease inhibitors (reviewed in refs. 56 and 67). In agreement with these 'promiscuous' ligand binding properties, LRP/CD91 has been assigned a multitude of physiologic functions (56, 67).

LRP/CD91 is expressed in many different cell types, including macrophages, hepatocytes, fibroblasts, adipocytes, neurons, and syncytiotrophoblasts (70). A particularly high expression is seen in macrophages of the anti-inflammatory phenotype that also express CD163. It is synthesized as a 600 kDa polypeptide that is cleaved by furin to form an 85 kDa fragment consisting of the transmembrane and cytoplasmic regions and a noncovalently attached 515 kDa extracellular region (34, 138). The extracellular region harbors 31 complement-type repeats (clustered in four domains as two, eight, ten, and eleven repeats), 23 EGF repeats, and eight so-called YWTD propeller domains (Fig. 6). The consecutive arrangement of two such EGF repeats followed by a YWTD propeller repeat and another EGF repeat, as observed in LRP/CD91 and the LDL receptor, is referred to as an EGF precursor homology domain.

The complement-type repeats are responsible for the binding of most LRP ligands. In contrast, the EGF repeats and YWTD propeller domains appear to mediate uncoupling of ligands at the low pH found in endosomes (56). The intracellular region comprises several motifs complying with consensus signals for internalization: two dileucine motifs and two NPxY motifs one of which overlaps with an YXXL motif. Of these, the latter is thought to constitute the dominant endocytic signal (55).

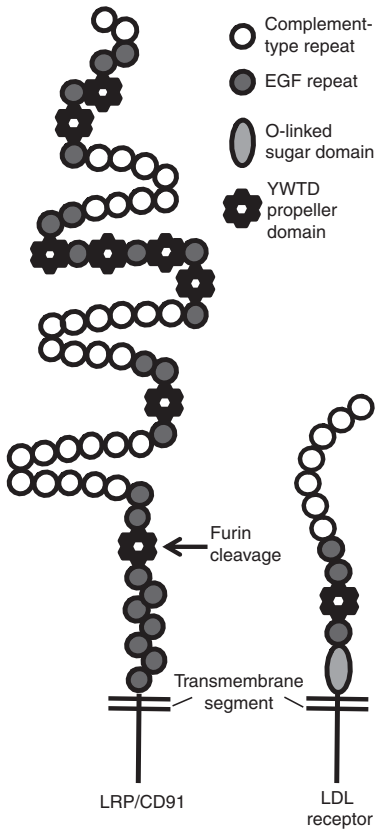

FIG. 6. Schematic representation of LRP/CD91 and the LDL receptor. LRP/CD91 and the LDL receptor share the following common motifs: complement-type repeats, EGF repeats, and YWTD propeller domains. The consecutive constellation of two EGF repeats followed by a YWTD propeller domain and yet an EGF repeat is referred to as an EGF precursor homology domain. The *arrow* indicates the position of cleavage by furin.

Mechanism of Hx-LRP/CD91-Dependent Scavenging

Experimental analyses have revealed striking similarities between the Hp-CD163-dependent and Hx-LRP/CD91-dependent scavenging systems of Hb and heme, respectively (42, 49, 100). First, the high affinity complex formed between heme and Hx elicits recognition by LRP/CD91 in a manner similar to the activation of CD163 binding upon Hp–Hb complex formation. Hence, both of these heme detoxification pathways work through plasma proteins that guide the toxic Hb/heme to receptors for endocytosis. Second, by analogy with the Hp–Hb-CD163 pathway, LRP/CD91 appears to segregate from its ligand Hx-heme in the early endosome and recycle to the membrane in accordance with classic receptor-mediated endocytosis (42, 100). Moreover, as was demonstrated for Hp, pulse-chase experiments revealed that Hx, in contrast to earlier observations (111), is degraded in the lysosome (42), thus explaining the observed decrease in plasma Hx concentrations in patients with severe hemolysis. Third, as is also observed upon Hp–Hb endocytosis by CD163 (90), internalization of Hx-heme by LRP/CD91 results in induction of HO-1 (42).

The Hx-LRP/CD91-dependent scavenging pathway may be considered an efficient 'backup' mechanism for removal of

heme once plasma Hp has been exhausted. The finding that Hp–Hx double knockout mice are more susceptible to hemolytic damage than wild-type and single Hp/Hx knockout mice is in perfect agreement with these somewhat overlapping roles of Hp and Hx in the resolution of hemolytic stress. Further, it underscores the importance of both Hp and Hx in protecting against undesirable effects of intravascular hemolysis (57, 58, 122, 123). The recently reported coexpression and glucocorticoid-mediated coregulation of CD91 and CD163 on human monocytes (64) is merely a further indication of the functional interrelationship between the Hp-CD163- and Hx-LRP/CD91-dependent scavenging pathways. Figure 7 illustrates the Hp–CD163- and Hx–LRP/CD91-dependent scavenging of 'free' Hb and heme, respectively.

Identification of the widely expressed LRP/CD91 as the Hx-heme receptor suggests that Hx-heme uptake may take place in a broad range of cell types. Of these, the hepatocytes and macrophages of the liver and spleen probably account for the majority of Hx-heme clearance. The relatively high Hx concentrations in the cerebrospinal fluid together with the high expression of LRP/CD91 in neurons furthermore suggest an important function for Hx-LRP/CD91-dependent heme clearance in the central nervous system. Combining this with the high expression of HO-1 in neurons, it is tempting to speculate that the concerted action of the Hx-LRP/CD91 endocytic system and HO-1 protects the neural tissue against oxidative stress subsequent to cerebral tissue damage and hemorrhage.

Other Heme-Binding Proteins

In addition to binding Hx, 'free' heme may bind the abundant plasma protein albumin and the serum lipocalin α_1-microglobulin. Heme binding by albumin is of low affinity and albumin probably constitutes a temporary destination for heme before transfer to the less abundant Hx (87). α_1-Microglobulin, on the contrary, plays a much more direct role in the defense against toxic heme due to its ability to degrade heme by means of an as yet unknown mechanism (3).

PCFT/HCP1 could also be speculated to play a role in uptake of 'free' heme. At any rate, PCFT/HCP1 is expressed in liver and kidney, both of which are involved in heme scavenging, as well as by macrophages (102), the major heme-scavenging cell type of the body. In agreement with a putative role for PCFT/HCP1 in heme scavenging, Sharma *et al.* recently suggested that the heme transport function of PCFT/HCP1 may extend beyond that of intestinal heme absorption to a more general role in heme trafficking across the plasma membrane of many different cell types (107). PCFT/HCP1 has other roles though. Recently, it has been shown to be the intestinal transporter of folate that apparently binds with an affinity that is approximately two orders of magnitude higher than that reported for heme (93, 108).

Therapeutic Perspectives

The finding that CD163 is solely expressed in monocytes/macrophages might be exploited to direct drugs specifically to monocytes/macrophages. Such specific targeting could be attained by chemical linkage of the drug in question to Hp–Hb complexes. Use of this treatment strategy could be beneficial in situations where CD163-expressing macrophages are involved in pathogenesis. These diseases include anti-inflammatory diseases, many infections, and certain cancers. Presently, initiatives in our laboratory are under way to address such treatment strategies.

Conclusions

The most important and well-described mechanism protecting against 'free' Hb is governed by the plasma protein Hp and the endocytic receptor CD163. Hp instantly captures Hb released into the circulation during intravascular hemolysis and directs it to macrophages expressing CD163. Binding of Hp–Hb to CD163 triggers endocytosis, and inside the cell the heme moiety is converted into nontoxic compounds. As a backup for this scavenging system, the plasma protein Hx and the receptor LRP/CD91 work together to capture and inter-

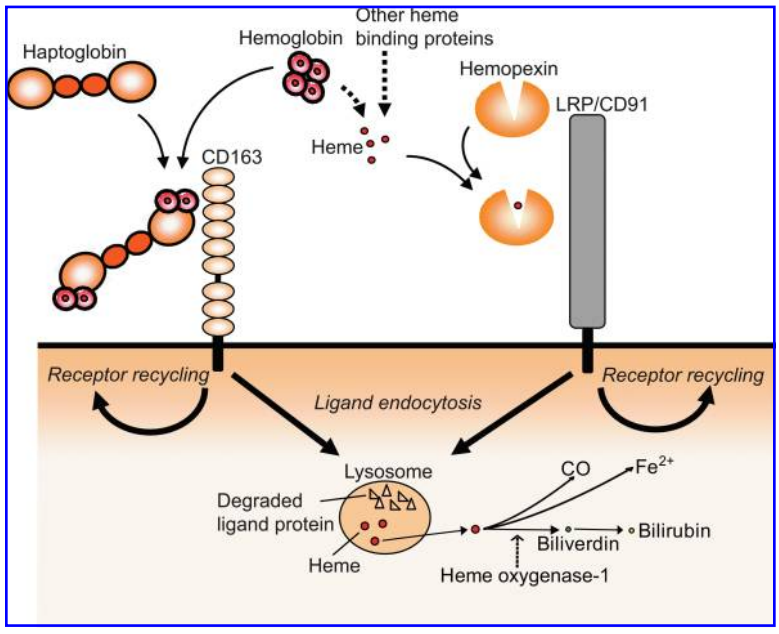

FIG. 7. Pathways for receptor-mediated endocytosis of 'free' Hb and heme complexed with Hp and Hx, respectively. Hb is cleared from the circulation in complex with Hp by the macrophage scavenger receptor CD163. The LRP/CD91-dependent scavenging of heme in complex with Hx functions as an efficient back-up mechanism for clearance of heme released from Hb and other heme-binding proteins. Upon endocytosis, the protein moieties of the ligands are degraded whereas the receptors recycle to the cell surface. Heme is converted to the overall anti-inflammatory molecules CO, Fe^{2+} and biliverdin/bilirubin by means of HO-1 and the biliverdin reductase in the cytosol.

nalize 'free' heme, a scavenging system that in many ways resembles the Hp-CD163 pathway for Hb clearance. In addition, other Hb/heme detoxification systems are thought to exist, thereby emphasizing the necessity to eliminate the highly toxic Hb/heme.

Importantly, heme scavenging not only protects against the undesirable effects of Hb and heme, but also helps maintaining iron homeostasis by allowing recycling of iron that would otherwise be lost in the urine. Moreover, limiting the amount of 'free' heme and Hb in plasma and other extracellular fluids is important in host defense against invading microorganisms that may require iron/heme for growth. Somewhat paradoxically to the general toxicity of Hb/heme released into plasma, recent research now shows that Hb in plasma may in fact have a beneficial function under certain circumstances, as exemplified by the critical role played by the Hpr-associated Hb in the primate innate defense against certain trypanosomes.

Continued research in receptor pathways for Hb/heme uptake is important to further our understanding of the basic mechanisms protecting against the undesirable effects of 'free' Hb/heme. Undoubtedly, future research within this field will uncover additional proteins involved in heme scavenging and it could furthermore perhaps help disclosing transport pathways for other tetrapyrroles.

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Author Disclosure Statement

SKM and HJM have declared ownership interests in Cytoguide, a spinoff company of The University of Aarhus. MJN declares no competing financial interests.

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Abbreviations Used

Hb = hemoglobin
HO-1 = heme oxygenase-1
Hp = haptoglobin
Hpr = haptoglobin-related protein
Hx = hemopexin
IL = interleukin
LDL = low density lipoprotein
LPS = lipopolysaccharide
LRP = low density lipoprotein receptor-related protein
NO = nitric oxide
PCFT/HCP1 = proton-coupled folate transporter/heme carrier protein 1
sCD163 = soluble CD163
SRCR = scavenger receptor cysteine-rich
TLF1 = trypanosome lytic factor 1
TLR = toll-like receptor

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