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News & Views

Gating the Radical Hemoglobin to Macrophages: The Anti-Inflammatory Role of CD163, a Scavenger Receptor

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ABSTRACT

Efficient extracellular hemoglobin (Hb) clearance is essential to prevent oxidative- and nitrosative-mediated toxicity. CD163 belongs to group B of the scavenger receptor cysteine-rich (SRCR) protein family found on the surface of monocytes and macrophages and is responsible for Hb-haptoglobin (Hp) complex uptake. Hb uptake by CD163 was thought to proceed exclusively through an Hp-dependent pathway. However, Hb can interact directly with CD163 via a low affinity binding when Hp is absent. As a result, a two-phase hypothesis of Hb clearance by monocytes/macrophages suggests that Hp–Hb binding to CD163 is the primary mechanism of plasma Hb clearance, while clearance of Hb by direct binding to CD163 is secondary to Hp depletion. The authors have considered the ligand specificity of CD163 in human macrophages and in a heterologous gene expression model to demonstrate that Hb is effectively endocytosed by CD163 in the absence of Hp. Additionally, the authors have considered Hb-based oxygen carriers (HBOCs) administration as a unique situation during which direct CD163 uptake may be relevant as a mechanism of clearance. However, the nature of chemical modifications introduced onto the Hb molecule and/or oxidative changes induced in the protein appear to influence the extent of CD163 interaction and cellular uptake. Here, an overview and novel insights into the role of CD163 in Hb redox inactivation and clearance are provided. *Antioxid. Redox Signal.* 9, 991–999.

INTRODUCTION

The oxygen carrier protein, hemoglobin (Hb), is one of the most abundant proteins in higher organisms. Intricate sequestration of Hb within red blood cells (RBCs) minimizes free Hb accumulation in high concentrations in plasma and, thus, limits its toxicity. When Hb is freed during hemolysis or as in the case with cell-free Hb-based blood substitutes, two major mechanisms of Hb toxicity, oxidative damage and hypertension, have been identified and intensively studied (1, 5, 40). Typically, haptoglobin (Hp) functions to efficiently bind and remove Hb released by normal RBC turnover or mild hemolysis via gating Hb to a high affinity receptor for Hb–Hp complexes identified as CD163 and found only on the surface of cells in the monocyte/macrophage lineage (24). Circulating plasma levels of Hp range from 0.45 to 3 mg/ml (24); however, when Hp concentrations are depleted during circumstances such as severe hemolysis, a second pathway involving direct interaction of Hb with monocyte/macrophage associated CD163 becomes operative (43). This may also be a relevant pathway of local tissue clearance when non-Hp binding Hb-based blood substitutes are administered in situations of local inflammation, sepsis, and trauma (43). The concept of a secondary low affinity pathway of Hb removal by monocyte/macrophages expressing CD163 and operating independent of Hp lends

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itself to a two-phase model of Hb clearance. The evolutionally processes which support Hp-independent action of CD163 in certain species will be discussed. The two-phase model is the primary topic of the present review; however, other recent work in this area suggests additional protective mechanisms that may contribute to detoxification of cell-free Hb. For example, we have recently reported a new intrinsic property of Hb characterized by a pattern of Hb β-globin chain peroxide mediated oxidation followed by heme/porphyrin α globin chain internalization/crosslinking (20). The role of hemopexin and its interaction with LRP/CD91 receptor complexes on monocytes and macrophages as well as direct binding of Hb to soluble CD163 represent potentially important contributions to the overall process of Hb detoxification (17). Since Hb-based blood substitutes are primarily extracellular, chemically modified and/or recombinant administered in large volumes for hemorrhagic shock and in smaller volumes for ischemic diseases and sepsis, it is likely that removal of this rather heterogeneous class of proteins from the circulation occurs in part via pathways common to those for extracellular Hb. We have found that chemical modification involving increased molecular size has an important influence on CD163 monocyte/macrophage-mediated uptake of polymerized Hb-based blood substitutes. Moreover, we have found a relationship between the type of chemical modification (*e.g.*, hydrophobic, hydrophilic, or charged) which enhance CD163 interaction and uptake by monocytes/macrophages (43).

HEMOGLOBIN-MEDIATED TOXICITY IN CELL-FREE ENVIRONMENT

Heme contains a highly reactive iron atom that easily participates in free radical-mediated redox reactions leading to oxidative modification of Hb and its transformation into a highly reactive species. Autoxidation of Hb results in the formation of non-oxygen binding ferric $HbFe^{3+}$ and superoxide ion ($O_2^{\bullet -}$), which can be dismutated into H_2O_2 and water. Catalase (CAT) and superoxide dismutase (SOD) within RBCs maintains reasonably low levels of $O_2^{\bullet -}$ and H_2O_2, respectively. However, during increased physiologic stress such as those induced by hemolysis, rhabdomyolysis, and ischemic conditions, levels of H_2O_2 may increase and accumulate in local tissue environments. With regard to free Hb, the end result is oxidative damage and loss of protein and heme structural integrity with formation of potentially toxic heme degradation products (Fig. 1). In addition, we have recently determined a highly reproducible pattern of Hb amino acid oxidation which occurs in the presence of H_2O_2 (20). Sub- and stoichiometric concentrations of H_2O_2:Hb consistently oxidize reactive sulfur-containing amino acids in the β globin chain as determined by mass spectrometry and amino acid analysis. βMet55, βCys93, and βCys112 undergo irreversible oxidation to methionine sulfoxide and cysteic acid. Additionally, βTrp15 was found to consistently oxidize to oxyindolyl and kynureninyl products (20). These findings were consistent with structural changes in the heme pockets and characterized by a decrease of α-helical content, heme loss, heme–protein linkages, and β globin chain collapse. Interestingly, internalization of heme or porphyrin products occurred primarily within the α globin chain. These heme–protein cross-links in human hemoglobin observed by us represent a novel protective mechanism to shield the heme groups from further oxidative degradation in a similar fashion to the action of other hemoproteins such as peroxidases in plants when faced with oxidative stresses (16) (Fig. 2).

Hb-mediated redox reactions have been implicated in the oxidative damage of endothelial cells (7). Further, plasma proteins, such as lipoproteins, undergo oxidative damage in the presence of free Hb that might thus represent a distinct pro-atherogenic factor (13). The pro-inflammatory effects that are exerted by free heme are likely related to the same redox processes (12, 19, 27, 48). The second mechanism of Hb toxicity relates to the ability of this molecule to bind to and inactivate the major vasodilator, nitric oxide (NO) (32). NO scavenging by free Hb negatively affects vasomotor balance toward inadequate vasoconstriction (Fig. 1). The physiological relevance of the latter process was impressively illustrated by two observations. First, patients with sickle cell disease—a hereditary chronic hemolytic disease—develop severe pulmonary hypertension that responds to treatment with inhaled NO (11, 38). Second, inadequate vasopressor activity and subsequent microcirculatory compromise are the major side effects of Hb-based blood substitutes that hamper their development as a safe alternative to blood (1).

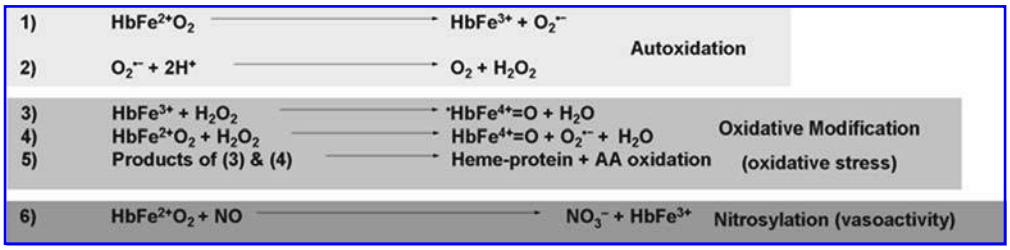

FIG. 1. Major toxicity mechanisms of free Hb. Oxidation and nitrosylation of free Hb lead to byproducts capable of tissue injury, and vasoconstriction implies that highly effective scavengers and detoxification systems must have evolved favorably for this molecule to function as an oxygen carrier and counter its properties as an oxidant and vasoconstrictor upon release from red blood cells.

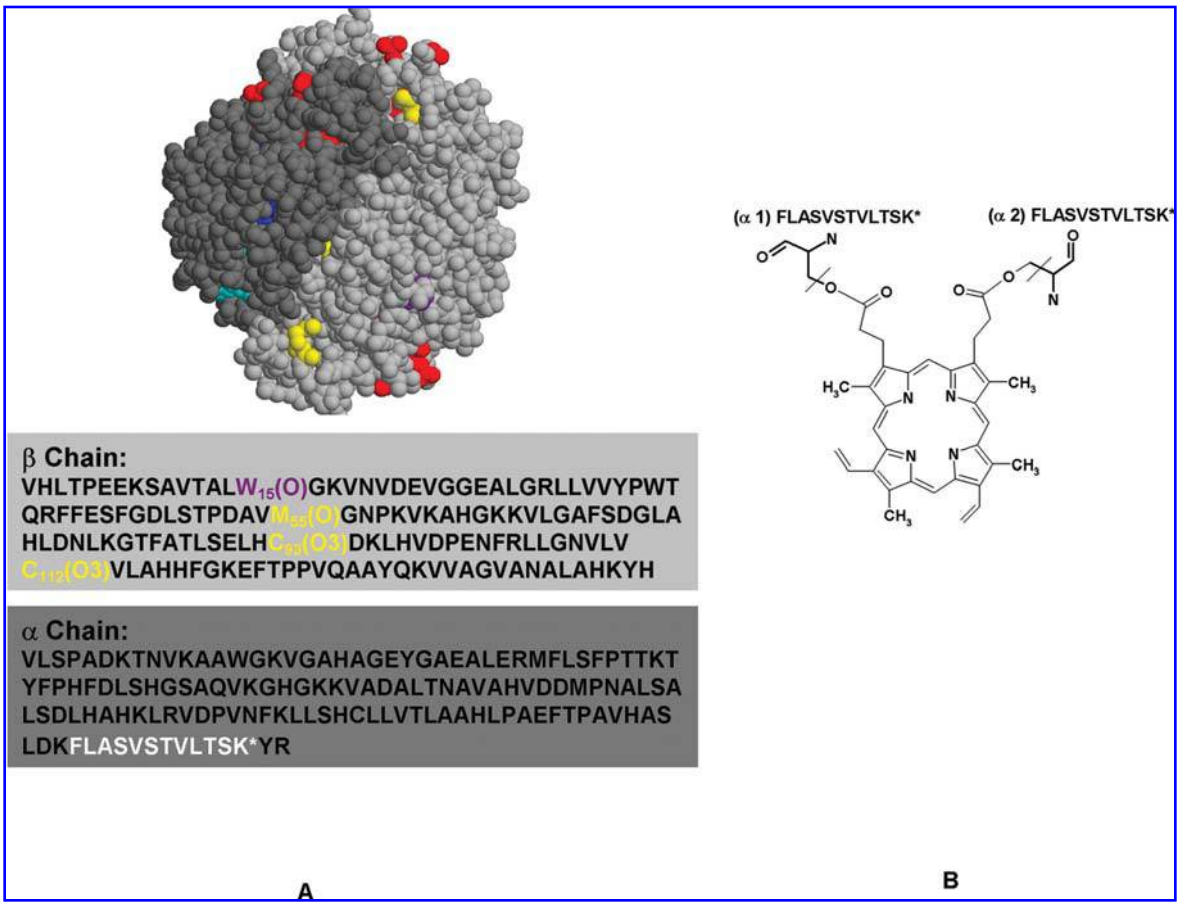

FIG. 2. Reactive oxygen species induced oxidation of Hb. Stoichiometric additions of H_2O_2 to Hb from 1:1 to 1:10 generate a highly reproducible pattern of amino acid oxidation limited to the β globin chain. The loss of β globin chain structural integrity appears to create protein internalization of heme/porphyrin cross-linkages between globin chains. In (**A**) the β globin chain is shown in *light gray,* and the α globin chain is shown in *dark gray*. The Hb sulfur containing amino acids found to undergo oxidation in the presence of H_2O_2 are shown in *yellow* within the β globin chain: *Yellow spheres,* (*top*) Cys112, (*middle*) Cys93, and (*bottom*) Met55, while the nonsulfur-containing Trp15 (*purple spheres*) was also found to reproducibly oxidize. The heme moieties are shown as *red spheres*. The amino acid sequence FLASVSTVLTS$_{138}$K shown in (**A**) as *cyan* and *blue* in the α1 and α2 globin chains were found to be cross-linked by a heme/porphyrin at the Ser138 positions as shown in (**B**). The PDB structure 1GZX was imported from the PDB into RasMol to generate the image and highlight single amino acids and amino acid sequence. The heme/porphyrin structure was generated in ISIS chem draw (MDL Information Systems, Inc., San Leandro, CA).

HAPTOGLOBIN AND CD163: A TWO-PHASE MODEL OF MACROPHAGE-MEDIATED HB CLEARANCE

Haptoglobin (Hp) is a liver-synthesized plasma protein that is highly conserved among animal species—including mammals, birds, snakes, turtles, and bony fish (14, 49). Hp binds free Hb with very high affinity and inhibits the ability of Hb to participate in redox reactions, in part through its intrinsic antioxidant activity (14, 31). Unlike other species, gene duplication has resulted in the existence of three distinct Hp phenotypes in humans (25). In contrast to the Hp 1-1 phenotype, which is synthesized as a protein dimer with two Hp monomers (each consisting of an Hp α and β-chain) linked by one disulfide bond between the two α-chains, the α-chain of the Hp 2-1 and Hp 2-2 phenotype has two reactive cysteine residues that allow the formation of disulfide bridges with two other α-chains. This property of Hp 2-1 and Hp 2-2 leads to the formation of a heterogeneous mixture of larger polymeric Hp molecules. When Hb is released into the circulation, it rapidly and irreversibly forms a complex with Hp. In its direct interaction with Hb, Hp impairs the filtration of the 32-kDa, small Hb dimer by the kidney, and likely limits the diffusion of free Hb into the intracellular spaces between endothelial cells and the vascular smooth muscle layer where Hb toxicity due to NO scavenging is expected to be the most deleterious. After its formation, the Hb–Hp complex is rapidly cleared from the circulation—a process that was

thought to occur largely within both the liver and spleen. In search of the endocytic receptor that mediates the clearance of Hb–Hp within the liver, Kristiansen *et al*. identified the macrophage-specific scavenger receptor, CD163, as the only high-affinity receptor for Hb–Hp complexes (24). Molecularly, CD163 belongs to group B of the scavenger receptor cysteine-rich (SRCR) protein family and is composed of nine highly homologous SRCR domains (39). In their initial studies, the authors postulated that only the Hb–Hp complex, but not either Hb or Hp alone, serves as a ligand for CD163. When Hb is bound to the multimeric Hp 2-2, it has a significantly higher affinity for CD163 than when bound to Hp 1-1; it thus seems likely that the individual Hp phenotype has a critical influence on free Hb clearance and Hb-related toxicity (2). However, several observations make Hp-independent macrophage clearance of Hb likely. Neither Hp knock-out mice nor patients with a genetic deficiency of Hp (ahaptoglobinemia) display any phenotypic signs related to deficient Hb or iron metabolism—an observation that is even more surprising as a particularly high number of ahaptoglobinemic individuals were found in certain African populations with a high prevalence of hemolytic diseases, such as malaria and sickle cell disease (10, 26). We have therefore considered the ligand specificity of CD163 in human macrophages, as well as in a heterologous gene expression model, and were able to show that Hb can be effectively endocytosed by the CD163 pathway even in the absence of Hp (43). Since the receptor–ligand affinity for free Hb is much lower than that for the Hb–Hp complex, the direct CD163–Hb interaction is expected to become a relevant Hb clearance mechanism after depletion of plasma Hp as it occurs either during massive hemolysis—or locally—after extravasation of large amounts of Hb during tissue injury. Recently, it was demonstrated that residues in β-chain loop 1 of Hp, particularly, Val-259, Glu-261, Lys-262, and Thr-264 are essential for the high-affinity binding of Hp–Hb to CD163 (kd = 12 n*M*) as compared to the low affinity binding of Hb to CD163 (kd = 0.4 μ*M*) (35). These data thus form the basis for our *two-phase model of plasma Hb clearance by CD163*. When only small amounts of Hb are released from bursting red blood cells (i.e., during physiological hemolysis), the free Hb binds to Hp and is rapidly cleared via high-affinity binding and subsequent endocytosis by CD163. However, once the amount of free Hb exceeds the plasma Hp-binding capacity, the low-affinity binding of free Hb to CD163 becomes the relevant pathway for Hb removal. The fact that at equal concentrations, uncomplexed Hb induces higher mRNA levels of the heme breakdown enzyme, heme oxygenase (HO-1), and also induces higher intracellular concentrations of the iron storage protein, ferritin, than does Hb bound to Hp may indicate that the low-affinity pathway may have a higher clearance capacity than does the high-affinity Hb–Hp clearance pathway (43, 44). We have shown that the high endocytic capacity of CD163 is, at least partially, related to the ability of the receptor to undergo constitutive and ligand-independent endocytosis with subsequent recycling to the cell surface (44). In this way, CD163 resembles other receptors that possess high endocytic capacity, such as the transferrin receptor or the low density lipoprotein receptor (LDLR).

Our previous data demonstrated that unlike Hb–Hp binding, which occurs through the α globin chain, a C-terminal amino acid sequence in the β globin chain is involved in direct uptake by CD163 on expressing cells (43). Thus, when Hb is released from RBCs and if exposed to conditions of oxidative stress described above, the following scenario can be envisioned (Fig. 3). After the collapse of the β chain in oxidatively damaged Hb, several potential mechanisms exist for Hb local tissue detoxification: heme/porphyrin internalization within the α globin chain to prevent further oxidative reactions, CD163-mediated scavenging of β globin and/or β globin–heme by macrophages. It remains to be determined, however, how oxidative changes on the Hb protein surface may ultimately affect the site-specific interactions between Hb, Hp, and CD163. In addition, other possible clearance mechanisms may also be operative: As reported recently, complexes of free heme and the soluble heme scavenger protein hemopexin are internalized by monocytes/macrophages via the low density lipoprotein receptor-related protein (LPR)/CD91 receptor (17). Given the physiologic importance of this process, it is likely that other low affinity receptors exist to remove Hb from the circulation. That Hb might be a ligand for multiple receptors is suggested by the low affinity Hb binding to the two epithelial receptors, cubilin and megalin, which

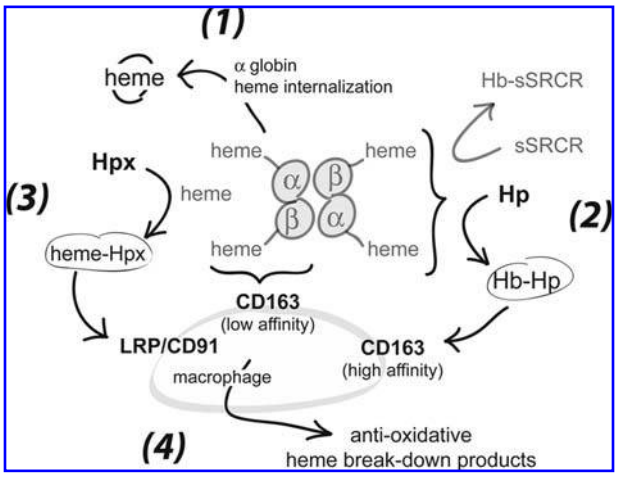

FIG. 3. Summary of physiologic Hb detoxification pathways. Four principle and interacting pathways are operative to remove Hb, heme, and its toxic byproducts after oxidative damage. ***(1) intrinsic Hb heme scavenging:*** Heme globin crosslinking and internalization within the Hb α chain shields heme from further oxidative damage. ***(2) soluble Hb scavenger proteins:*** Within plasma Hb α/β dimers are bound by Hp. Soluble SRCR domain proteins that avidly interact with free Hb to form stable complexes have been characterized in chicken plasma and several candidate proteins have been identified in mammals. ***(3) soluble heme scavenger proteins:*** Free heme is avidly bound to the plasma protein hemopexin (Hpx). ***(4) macrophage clearance of heme and Hb:*** Free Hb is edocytosed by the macrophage Hb scavenger receptor through low affinity interaction of CD163 with a binding site on the c-terminal β globin chain. Hb–Hp complexes are cleared through high affinity binding to CD163. Heme–Hpx complexes are also cleared by macrophages after binding to LRP/CD91.

might play a role in Hb/iron recovery after filtration of Hb dimers by the kidney (9).

EVIDENCE FOR AN INTRAVASCULAR HB CLEARANCE (CIRCULATING CD163/MONOCYTES)

Based on our current understanding, Hb–Hp complexes are thought to be cleared by spleen and liver macrophages that express particularly high levels of CD163. However, according to this model of free Hb clearance, the passage of Hb from the site of erythrocyte destruction (anywhere within the circulation) to the hepatic/splenic circulation would require considerable time. Since both NO-scavenging and heme-oxidative reactions are fast and irreversible, an intravascular Hb clearance pathway that acts at the site of erythrocyte destruction could significantly reduce the circulation time of free Hb and thus limit the physiological side effects of free Hb. Using a new, whole blood assay for cellular Hb–Hp endocytosis, we have examined whether peripheral blood leukocytes could serve as putative intravascular Hb clearance compartments (45). Hb–Hp endocytosis is a functional property of a distinct leukocyte subpopulation that could be unequivocally identified as monocytes, based on their light-scatter patterns and high level expression of both CD14 and CD64. We did not observe uptake of any Hb–Hp complexes by either granulocytes or lymphocytes.

Expression of the Hb scavenger receptor, CD163, is highly induced during the differentiation of peripheral blood monocytes into mature macrophages. However, whether circulating monocytes express significant levels of CD163 is a topic of debate. We have therefore examined whether monocytes express this candidate Hb clearance receptor. Since monocyte autofluorescence is virtually absent in the far-red allophycocyanin (APC) excitation/emission range, we used a novel, APC-conjugated monoclonal antibody to CD163 to significantly enhance the signal-to-noise ratio of our FACS analysis. Our results provide definitive proof that all $CD14^{high}$/ $CD64^{high}$ monocytes also express CD163. The fact that the Ca^{2+}-dependent (28) Hb–Hp uptake by monocytes was abolished by the addition of excess α–α-cross-linked tetrameric Hb (α–α DBBF-Hb)—which is an Hp-independent CD163 ligand (43)—suggests that a receptor-dependent mechanism is involved in monocyte Hb–Hp uptake. Further, CD163 seems to play a crucial and possibly nonredundant role in the clearance of free Hb in circulating blood since monocyte Hb–Hp uptake was completely inhibited by a polyclonal antibody that was previously shown to block the Hb–Hp interaction with CD163. With these experiments, we could demonstrate that CD163 expressed by peripheral blood monocytes serves as an Hb clearance pathway that is active at the site of red cell destruction within the vascular bed.

HB–CD163 HEME OXYGENASE PATHWAY: THE ANTI-INFLAMMATORY ROLE OF CD163

CD163-positive macrophages have been thought to possess an anti-inflammatory function. This association is primarily based on the following observations. First, macrophages highly expressing CD163 constitute the predominant macrophage population during the late or resolution phase of inflammatory reactions (50). Second, CD163 expression is strongly induced by anti-inflammatory mediators, such as glucocorticoids and IL-10 (15, 41, 46). We have proposed that CD163 may exert anti-inflammatory function in part by the removal of toxic Hb from damaged tissue, a function that is induced by glucocorticoid treatment of human macrophages *in vitro* (42). To further understand the macrophage response to Hb and also to unravel novel anti-inflammatory pathways linked to CD163, we have performed global gene expression analysis of macrophages treated with highly purified and endotoxin-free Hb (44). Beyond the finding that transcriptional macrophage response to Hb is strictly noninflammatory, our findings also suggest that induction of the inducible heme breakdown enzyme, heme oxygenase (HO-1), is the principle response. Accordingly, high intracellular levels of the iron storage protein, ferritin, accumulate in Hb-treated macrophages—a process that was proven to relate to enzymatic HO-1 activity. In a heterologous CD163-expression model in HEK293 cells, our data further revealed that HO-1 induction by Hb is dependent on functional CD163 expression and that internalization of heme, but not receptor phosphorylation-dependent signaling, exerts the critical trigger of HO-1 transcription.

The ability of CD163 to mediate upregulation of HO-1 expression and subsequent synthesis of protective compounds, such as ferritin, in response to extracellular Hb may point to unrecognized roles for Hb and CD163 in mediating an anti-inflammatory and wound healing macrophage phenotype in monocytes after infiltration of damaged tissues. HO-1 is the rate-limiting enzyme in the catabolism of heme. The byproducts of heme breakdown include carbon monoxide and bilirubin, as well as free iron, which is rapidly sequestered by ferritin. Carbon monoxide, bilirubin, and ferritin have all been assigned a multitude of antioxidative and anti-inflammatory properties that have been demonstrated to be of particular relevance in wound healing and as protective factors in the pathogenesis of atherosclerosis (33, 36, 37, 47). Our finding that CD163-positive macrophages constitute the major compartment of HO-1 expression in human atherosclerotic lesions not only lends evidence to the limitation of Hb toxicity through Hb clearance by CD163-positive macrophages within the atherosclerotic vessel wall, but also to the operation of the Hb/CD163/HO-1 pathway *in vivo*. Figure 4 illustrates the Hb/CD163/HO-1 pathway as an anti-inflammatory macrophage effector pathway.

PHYLOGENETIC EVIDENCE FOR A CONSERVED ROLE OF THE SRCR DOMAIN HB SCAVENGER

Haptoglobin displays a wide range of cross-species interactions with Hb from different animals. In the late 1970s, a high-affinity functional analog of Hp was also described and characterized in chickens (34). “Chicken Hp” is a soluble and acute phase-inducible plasma protein, as is its mammalian analog, exerting a very high affinity for chicken Hb. However, whereas chicken Hb avidly interacts with mammalian Hp, “chicken Hp” only weakly, if at all, forms complexes

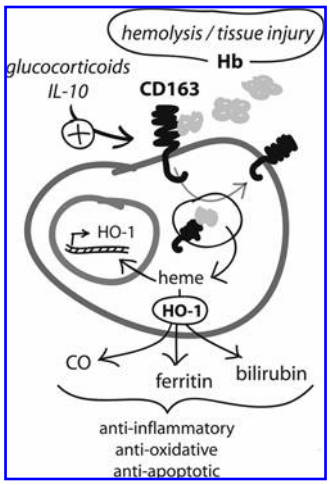

FIG. 4. The Hb/CD163/HO-1 pathway as an anti-inflammatory macrophage effector pathway. During hemolysis or at sites of tissue injury, free Hb is released from red blood cells and endocytosed by CD163 expressing monocytes/macrophages. Internalization of Hb–heme via constitutive endocytosis of CD163 is the major trigger of HO-1 expression in Hb-exposed macrophages. The heme breakdown products carbon monoxide (CO), bilirubin, and ferritin are supposed to mediate the anti-inflammatory effects of the CD163 positive macrophages.

with mammalian Hbs (34). This finding led us to conclude that, although the Hp-binding site on Hb appears to be highly conserved during evolution, considerable variability must exist between Hp molecules from different species. Surprisingly, "chicken Hp" was recently found to not be related to mammalian Hp but instead comprises a novel SRCR protein family member called pit54 (also called 18-B) (18, 49). Notably, as chicken have no Hp gene, pit54 is the only Hb scavenger found in chicken plasma.

Pit54 is composed of four SRCR domains that have high protein sequence similarity to the mammalian Hb scavenger receptor, CD163, and also to the related mammalian group B SRCR proteins, such as CD6 and DMBT1 (deleted in malignant brain tumors 1), and the two soluble proteins, SP-alpha and S4D-SRCRB. The identification of pit54 as the exclusive high-affinity, Hb-binding protein in chicken plasma, together with the high sequence similarity found between pit54 and the only other known SRCR Hb-binding protein, CD163, lends evidence to the hypothesis that the SRCR domain has an ancient and highly conserved role in hemoglobin binding and detoxification. To prove this hypothesis, it will be important to further examine the Hb-binding properties of the highly homologous SRCR domains of pit54, CD163, SP-alpha, S4D-SRCRB, and CD6. The close relationships between CD163, pit54, and the soluble mammalian SRCR proteins, SP-alpha and S4D-SRCRB, might point towards a role for these proteins as alternative soluble Hb scavengers in mammals that might have functional relevance in states of Hp depletion, such as hemolysis, or in individuals with genetic ahaptoglobinemia, a genotype that does not display any phenotypic changes that are attributable to increased systemic Hb toxicity (8).

HEMOGLOBIN-BASED OXYGEN CARRIER CLEARANCE AND CD163

Chemical and recombinant technologies employed in the production of Hb-based oxygen carriers (HBOCs) "blood substitutes" are used to stabilize and/or increase the molecular size of the Hb tetramer. As a result, HBOCs display differing circulatory persistence compared to native extracellular Hb (6, 21–23). Modification to Hb alters its overall pharmacokinetic parameters through both chemical changes in the protein (*e.g.*, tetramer stabilization, polymerization, and surface conjugation) as well as physical properties (*e.g.*, viscosity and colloid osmotic pressure) (4). HBOCs are minimally eliminated in animals via renal clearance (≤5%) following infusion of either stabilized tetramer or polymer (3). A ^{99m}TC-labled HBOC was found to distribute in small quantities to extracellular compartments and via transcapillary exchange followed by lymphatic drainage (4). The extent of transcapillary exchange is believed to be influenced by HBOC molecular size with smaller tetrameric forms being eliminated faster than polymers (29, 30). Numerous mechanisms appear to contribute toward circulating HBOC removal and these processes suggest an extremely high threshold for elimination. The mechanisms of HBOC clearance are not fully understood, however, it is likely that liver, spleen, and kidney tissue are all responsible for metabolic breakdown of HBOCs-based distribution studies (4, 22). The type of modification and resulting chemical and physical properties imparted on the protein also influence the overall process. HBOCs are typically used as large quantity infusions in animals and humans, thus exceed gram/kg exposures. The plasma concentrations generated by these doses mimic or go beyond Hb exposures during severe hemolytic disease. Thus, unlike native extracellular Hb, HBOCs likely behave quite differentially in the previously described two-phase clearance model. Most HBOCs appear to demonstrate minimal binding to Hp even when Hp stores are not yet saturated; therefore, the primary route of clearance via macrophages is likely through direct interaction with CD163 (43). Our data suggests uptake of HBOCs by CD163 expressing macrophages is (a) independent of Hp, (b) dependent on HBOC molecular weight, and (c) can be influenced by chemical modification. With increased exposure to circulating HBOCs, it is possible that the risk for toxicity is also increased. HBOCs function most effectively as bridging mechanism to provide oxygen carrying capability when whole blood or packed red cells are not available. As a result, an HBOC with a limited circulating half-life which may be site specifically modified for removal through either the Hb–Hp–CD163 or Hb–CD163 pathway may be an attractive protective mechanism. Monocyte/macrophage uptake of HBOCs is likely not a major route of clearance from the systemic circulation; however, a more likely role for removal of HBOCs by CD163 expressing monocytes/macrophages is from tissue microenvironments where local oxidative and inflammatory processes take place. While speculative, the Hp-independent interaction of CD163 with HBOC may prove critical for limiting tissue injury induced

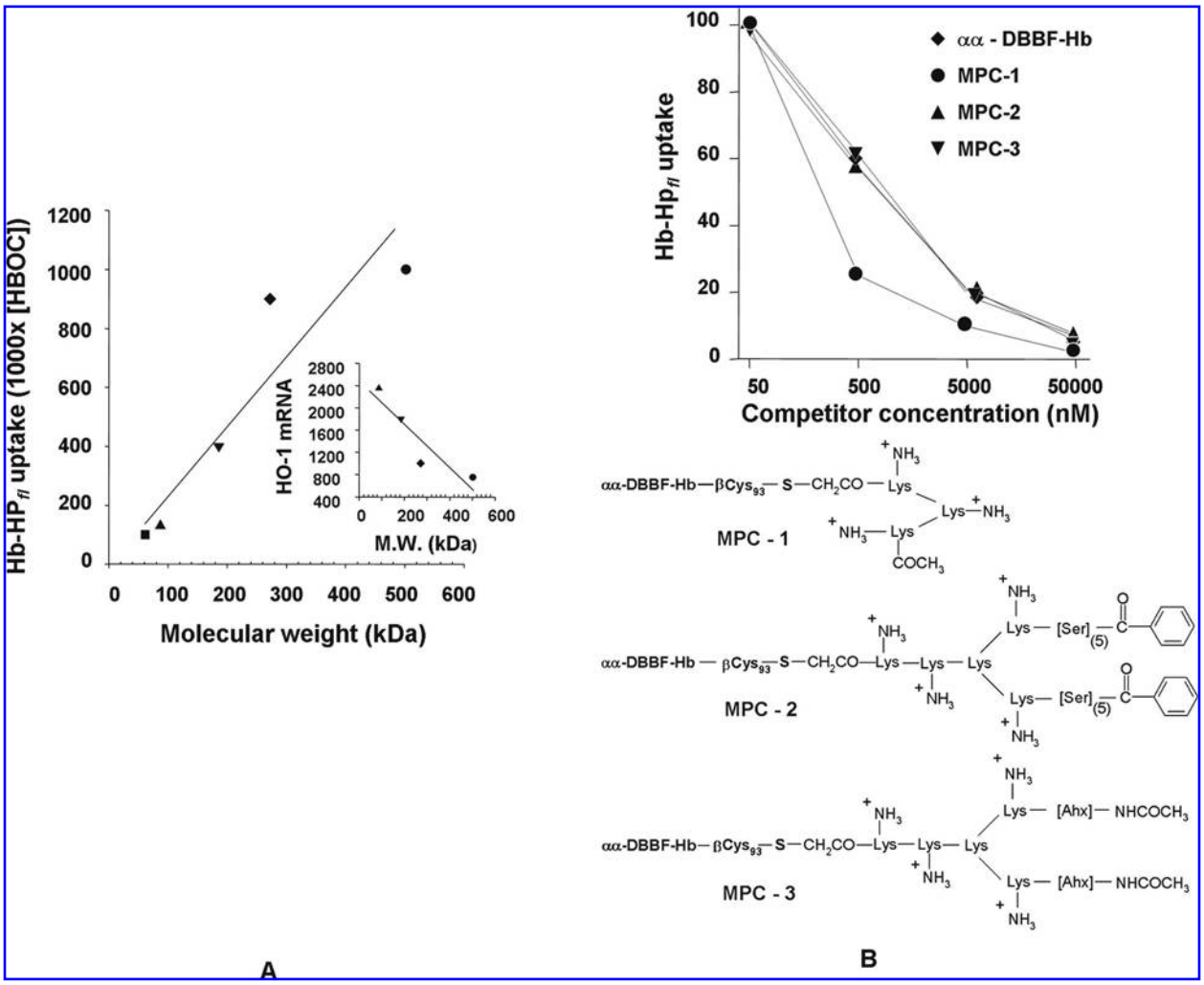

FIG. 5. CD163-mediated uptake and hemoglobin-based oxygen carriers. The relationship between HBOC M.W. and CD163 mediated uptake of a fluorescent Hb–Hp$_{fl}$ complex is shown in (**A**) when HBOC obtained from size exclusion chromatography fractions of glutaraldehyde polymerized bovine Hb was added at 1000x. The symbols represent differing M.W. HBOC fractions as follows: (■) 64 kDa, (▲) 87 kDa, (▼) 186 kDa, (◆) 272 kDa, and (●) 502 kDa. HBOC fraction M.W. was well correlated to Hb–Hp$_{fl}$ uptake ($r^2 = 0.874$). The inset in (**A**) shows the same M.W. fractions and their influence on CD163 expressing cells HO-1 expression, similarly a strong correlation exists between HBOC M.W. and HO-1 expression ($r^2 = 0.833$) . (**B**) demonstrates the influence that chemical modification of Hb has on enhancing CD163-mediated cellular uptake. A multiple peptide conjugate (MPC) approach to modification at αα-DBBF-Hb's βCys93 was used to prepare three chemical modifications: charged side chain (MPC-1), hydrophilic side chain (MPC-2), and hydrophobic side chain (MPC-3). The structures for MPC-1, 2, and 3 were generated in ISIS chem draw.

by HBOCs following conditions of ischemia/reperfusion. Figure 5 shows the relationship between molecular weight and CD163-mediated cellular uptake, as well as the influence of chemical modification to Hb as it relates to CD163 mediated uptake and clearance. This illustrates that a smaller stabilized Hb tetramer; unlike much larger molecular weight (M.W.) stabilized multi-tetramers can be scavenged by CD163 expressing cells. Competition for CD163 uptake of fluorescently labeled Hp–Hb as well as HO-1 expression in CD163 expressing cells are highly correlated with HBOC M.W. from 64 to 500 kDa. Interestingly, we also found that surface manipulation of the Hb molecule to bear clusters of lysine residues, mimicking part of amino acid sequence essential for Hp–Hb interaction, can promote the uptake of the same tetramer by the high affinity pathway. This may ultimately provide a useful strategy in which oxidatively prone Hb species can be readily and rapidly cleared by CD163–Hp system.

ABBREVIATIONS

APC, allophycocyanin; CAT, catalase; DMBT, deleted malignant brain tumors; FACS, fluorescent activated cell sorter; Hb, hemoglobin; HBOC, hemoglobin based oxygen

carrier; Hb–Hp, hemoglobin–haptoglobin complex; H_2O_2, hydrogen peroxide; HO-1, heme oxygenase -1; Hp, haptoglobin; LDR, low density lipoprotein receptor; M.W., molecular weight; NO, nitric oxide; $O_2^{\bullet-}$, superoxide anion; RBCs, red blood cells; SOD, superoxide dismutase; SP-alpha, soluble protein alpha; SRCR, scavenger receptor cysteine-rich; S4D-SRCRB, scavenger receptor super family group b protein; TC, technetium.

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