A novel hemoglobin-binding peptide reduces cell-free hemoglobin in murine hemolytic anemia

Madelyn S. Hanson,1,* Hao Xu,2,3,* Timothy C. Flewelen,1 Sandra L. Holzhauer,2,5 Dawn Retherford,2,5 Deron W. Jones,3 Anne C. Frei,5 Kirkwood A. Pritchard, Jr.,2,3 Cheryl A. Hillery,2,4,5 Neil Hogg,1 and Nancy J. Wandersee2,4,5

1Department of Biophysics and Redox Biology Program, Medical College of Wisconsin, Milwaukee, Wisconsin; 2Translational Vascular Biology Program, Medical College of Wisconsin, Milwaukee, Wisconsin; 3Department of Pediatric Surgery, Medical College of Wisconsin, Milwaukee, Wisconsin; 4Department of Pediatrics and Children’s Research Institute, Medical College of Wisconsin, Milwaukee, Wisconsin; 5Blood Research Institute, Milwaukee, Wisconsin; and Blood Center of Wisconsin, Milwaukee, Wisconsin

Submitted 28 June 2012; accepted in final form 29 October 2012

Hanson MS, Xu H, Flewelen TC, Holzhauer SL, Retherford D, Jones DW, Frei AC, Pritchard KA Jr, Hillery CA, Hogg N, Wandersee NJ. A novel hemoglobin-binding peptide reduces cell-free hemoglobin in murine hemolytic anemia. Am J Physiol Heart Circ Physiol 304: H328–H336, 2013.—Hemolysis can saturate the hemoglobin (Hb)/heme scavenging system, resulting in increased circulating cell-free Hb (CF-Hb) in hereditary and acquired hemolytic disease. While recent studies have suggested a central role for intravascular hemolysis and CF-Hb in the development of vascular dysfunction, this concept has stimulated considerable debate. This highlights the importance of determining the contribution of CF-Hb to vascular complications associated with hemolysis. Therefore, a novel Hb-binding peptide was synthesized and linked to a small fragment of apolipoprotein E (amino acids 141–150) to facilitate endocytic clearance. Plasma clearance of hE-Hb-b10 displayed a rapid phase t1/2 of 16 min and slow phase t1/2 of 10 h, trafficking primarily through the liver. Peptide hE-Hb-b10 decreased CF-Hb in mice treated with phenylhydrazine, a model of acute hemolysis. Administration of hE-Hb-B10 also attenuated CF-Hb in two models of chronic hemolysis: Berkeley sickle cell disease (SS) mice and mice with severe hereditary spherocytosis (HS). The hemolytic rate was unaltered in either chronic hemolysis model, supporting the conclusion that hE-Hb-B10 promotes CF-Hb clearance without affecting erythrocyte lysis. Interestingly, hE-Hb-B10 also decreased plasma ALT activity in SS and HS mice. Although acetylatedcholine-mediated facialis artery vasodilatation was not improved by hE-Hb-B10 treatment, the peptide shifted vascular response in favor of NO-dependent vasodilation in SS mice. Taken together, these data demonstrate that hE-Hb-B10 decreases CF-Hb with a concomitant reduction in liver injury and changes in vascular response. Therefore, hE-Hb-B10 can be used to investigate the different roles of CF-Hb in hemolytic pathology and may have therapeutic benefit in the treatment of CF-Hb-mediated tissue damage.

sickle cell disease; hereditary spherocytosis; hemolysis; mouse models

INTRAVASCULAR HEMOLYSIS results in elevated levels of cell-free hemoglobin (CF-Hb) in the plasma compartment. Normally, CF-Hb is rapidly bound by the scavenger protein haptoglobin, maintaining nominal levels of CF-Hb in the plasma (5, 31, 41). However, in chronic hemolytic diseases, this scavenging mechanism becomes saturated, allowing CF-Hb levels to increase in the circulation (5, 38, 49). CF-Hb possesses peroxidase-like activity that contributes to its cytotoxic properties by oxidizing lipids and generating prooxidative and proinflammatory products (5, 36, 48). CF-Hb also reacts directly with nitric oxide (NO), decreasing the bioavailability of NO as well as the vasodilatory and anti-inflammatory properties of this gaseous molecule (23, 49, 50). Together, these findings imply that elevated levels of CF-Hb may have a direct role in the oxidative and inflammatory injuries observed in hemolytic disease.

Indeed, intravascular hemolysis has been linked to pulmonary hypertension (19, 24, 26, 45, 59), systemic vasculopathies (27, 28, 50, 52, 53, 60), and cardiovascular complications (11, 25, 30, 39). Although such studies have suggested that CF-Hb likely plays important roles in vascular disease, the actual contribution of CF-Hb to disease pathology has drawn controversy, particularly in sickle cell disease (SCD) (9, 18). The release of intraerythrocyte arginase (37), erythrocyte and leukocyte adhesion (22, 42, 57), increased coagulant activity (10), and inflammation and reperfusion injury (21) are all potential contributors to the SCD pathology induced or aggravated by hemolysis. Therefore, distinguishing the role of CF-Hb in hemolytic pathology from other potential factors requires the development of agents that specifically target CF-Hb.

An obvious candidate for targeting CF-Hb is haptoglobin. The covalent Hb/haptoglobin complex has long been considered the major pathway for directing CF-Hb clearance (1, 5, 41). Although studies (3, 8, 33) have demonstrated that haptoglobin limits Hb-induced hypertension and renal damage, a recent study (3) showed that haptoglobin did not decrease the half-life (t1/2) of CF-Hb in the circulation or inhibit the ability of CF-Hb to react with NO. Additionally, CF-Hb clearance in haptoglobin-null mice was not altered compared with wild-type mice (32). These findings demonstrate that while haptoglobin likely provides some protection against Hb-induced injury, the capacity of haptoglobin to efficiently clear elevated levels of CF-Hb may be limited. Thus, novel Hb-binding agents that facilitate the clearance of CF-Hb are required to accurately address the role of CF-Hb in hemolytic pathology.

Here, we describe a novel Hb-binding peptide, hE-Hb-B10, that both binds Hb and clears it from the circulation. We examine its potential use as a novel approach to address the role of CF-Hb in hemolytic disease pathology.
MATERIALS AND METHODS

Human subjects. The use of human subjects was approved by the Institutional Review Boards of Children’s Hospital of Wisconsin, Medical College of Wisconsin, and BloodCenter of Wisconsin. Informed consent was obtained from healthy volunteers and individuals with homozygous Hb SS disease and/or guardians for minor children, with consent when appropriate. Individuals with a history of red blood cell transfusion within 2 mo before blood collection were excluded from the study.

Mice. C57BL/6J mice were purchased from The Jackson Laboratory (stock no. 000664, Bar Harbor, ME). Berkeley SCID mice [Tg(Hu-miniLCRA)1γ γΔ6 bp] HbaA1c/HbaA1c HbbA1c/HbbA1c, SS mice] are on the Berkeley mixed genetic background, exclusively express human sickle hemoglobin, and have a phenotype that mimics many features of severe SCID in humans (35, 44). Berkeley HbA mice [Tg(Hu-miniLCRA)1γ γΔ6 bp] HbaA1c/HbaA1c HbbA1c/HbbA1c, AA mice] are on the same Berkeley mixed genetic background as SS mice and exclusively express normal human Hb A. WBB6F1-sph/sph mice have severe autosomal recessive hereditary spherocytosis due to a spontaneous single-base deletion in the murine erythroid α-spectrin gene (Splen) (16, 55). The sph mutation is maintained in the heterozygous state on both the WB/ReJ (WB) and C57BL/6J (B6) backgrounds. F1 hybrid (WBB6F1) mutant mice (sph/sph; HS mice) and normal mice (++/+, sph/+; Ctrl mice) were generated by mating WB and B6 heterozygotes and are genetically identical except at the mutated locus. F1 hybrid mice survive longer than mutant mice on inbred strain backgrounds (16). Mice were cared for according to the Association for Assessment and Accreditation of Laboratory Animal Care specifications. Animal experiments were approved by the Institutional Animal Care and Use Committee of the Medical College of Wisconsin. Experimental groups contained similar numbers of male and female mice and were 6 wk of age or older.

Peptide identification and synthesis. Human Hb (10 nM, Sigma-Aldrich, St. Louis, MO) was coated onto a 96-well plate overnight followed by blocking with BSA and incubation with phage using phage display library PhD12 and following the manufacturer’s guidelines (New England Biolabs, Ipswich, MA). Unbound phage were washed away, and bound phage were harvested by elution with 1 mM HCl and amplified for further screening. Three rounds of selection were performed with increasingly stringent conditions by washing with 0.1, 0.2, and 0.3% Tween-20 at each successive round to enrich for phage with higher binding affinity for hemoglobin. We then amplified phage from single plaques for DNA sequencing and Hb-binding verification by ELISA-based assay where a microtiter plate was coated with hemoglobin, blocked with BSA, 10^10 to 10^12 virons added to each well, and the binding virons detected by HRP-conjugated anti-M13 antibody. We selected peptide Hb-B10 as our lead peptide. Hb-B10 was NH2-terminally coupled to LRKLRKKRLL, corresponding to amino acid residues 141–150 of human apolipoprotein E (ApoE), creating hE-Hb-B10 (13, 14). Peptides were synthesized using standard protocols on an ABI 433 (Applied Biosystems, Carlsbad, CA). The peptide resin was cleaved, precipitated, and lyophilized, followed by HPLC purification. The mass of the final product was verified by MALDI-TOF mass spectrometry analysis.

Binding kinetics. The NH2-terminus of Hb-B10 was biotinylated with EZ-Link NHS-LC-biotin (Thermo Fisher Scientific, Rockford, IL) and loaded onto streptavidin-coated biosensors at 25 μg/ml (Octet RED96 System, ForteBio, Menlo Park, CA). Biosensors were equilibrated in kinetic buffer (1X, ForteBio) and transferred to a solution of oxyHb (5–50 μM) to allow association between Hb-B10 and Hb. Biosensors were incubated in kinetic buffer without Hb to measure the dissociation of Hb from Hb-B10. Dissociation constants were calculated with data analysis software (version 6.3) from ForteBio.

NO scavenging assay. Washed streptavidin-coated magnetic beads (2.4 nmol/mg binding capacity, Solulink, San Diego, CA) were blocked in 10% normal human plasma in PBS for 20 min at 25°C. After being blocked, beads were incubated with plasma from individuals with SCID plus biotinylated Hb-B10 at 25°C for 10 min. Beads were pelleted from the sample using a magnet, and the supernatant was used in NO consumption experiments performed as previously described (58) with minor modifications. Briefly, 50 mM dipropylenetriamine NONOate (Cayman Chemical, Ann Arbor, MI) was added to 5 ml PBS and equilibrated in a purge vessel connected to a NO chemiluminescence analyzer. The chemiluminescence signal, reflective of NO within the chamber, was allowed to reach a baseline (100–150 mV), and samples (10 μl) were injected into the solution. A decrease in the chemiluminescent signal indicated consumption of NO by oxyHb or other plasma components. NO consumption by SCID plasma samples was quantified by comparing changes in the chemiluminescent signal to those generated by known concentrations of oxyHb.

Peptide clearance measurements. For plasma clearance experiments, hE-Hb-B10 was conjugated to 5,6-carboxyfluorescein (FAM; AnaSpec, Fremont, CA). C57BL/6J mice were injected with FAM-labeled hE-Hb-B10 (70 μg/mouse ip). For each time point, plasma was obtained from two deeply anesthetized mice as described below, and the fluorescence within the plasma was measured using a Wallac VICTOR counter (Perkin-Elmer Wallac, Waltham, MA, excitation: 488 nm/emission: 520 nm). Background fluorescence was determined in plasma from nontreated mice, and this value was subtracted. Arbitrary fluorescence values were normalized to a standard curve of FAM-labeled hE-Hb-B10 in mouse plasma to obtain micrograms per milliliter of peptide. These values were then fitted to a double exponential equation to determine t1/2 values of hE-Hb-B10 in plasma (Sigma Plot software, Systec Software, San Jose, CA) (17, 20).

Peptide tissue distribution. Mice were treated with FAM-labeled hE-Hb-B10 (70 μg/mouse ip) and harvested as described below. After perfusion of the mouse with PBS, the liver, kidney, spleen, lung, aorta, and brain were dissected and fixed in 4% paraformaldehyde for 24 h followed by cryopreservation in 30% sucrose at 4°C for 72 h. Tissues were mounted in OCT embedding media (Tissue-Tech, Torrance, CA) on dry ice and stored at −80°C. Frozen tissue sections on slides were fixed in 4% paraformaldehyde, washed in PBS, and stained with 4’,6-diamidino-2-phenylindole for 1 min (Invitrogen, Grand Island, NY). Stained slides were washed with PBS and mounted in fluorescence mounting medium (Vector Laboratories, Burlingame, CA). Slides were analyzed using an Olympus Fluoview FV1000 MPE multiphoton laser scanning microscope at ×20 or ×60 magnification (Olympus, Center Valley, PA).

Phenyldiazirine and peptide treatments. To induce acute hemolysis, C57BL/6J mice were given a single intraperitoneal injection of 4 mg/mouse phenyldiazirine (PHZ; Sigma) or PBS (32). PHZ-injected mice received hE-Hb-B10 (40 μg/mouse ip) or PBS at 10 and 21 h after PHZ. SS and HS mice received daily treatments with hE-Hb-B10 (10–20 μg/mouse ip) or PBS for 3 wk. All mice were harvested within 1 h of the final hE-Hb-B10 or PBS injection.

Whole blood and plasma assays. Human blood samples were collected into 3.8% sodium citrate (volume: 1:9). Murine blood was obtained from deeply anesthetized mice by cardiac puncture and drawn into anticoagulant (sodium citrate or heparin). Blood cells were separated from plasma by centrifugation at 2,000 g for 10 min. Plasma was further clarified by centrifugation at 8,100 g for 10 min and then aliquoted for storage at −80°C (16). A complete blood count was determined using an automated veterinary blood count reader (Heska, Fort Collins, CO). Reticulocyte counts were determined by flow cytometry using thiazole orange (BD Biosciences, San Jose, CA) based on the percentage of thiazole orange-positive cells within the erythrocyte gate (35). CF-Hb levels in plasma were measured using reagents from Catachem (Oxford, CT). Plasma lactate dehydrogenase (LDH) activity was assayed using a kit from BioAssay Systems (Hayward, CA). Plasma alanine aminotransferase (ALT) activity was quantified using a kit from Bioo Scientific (Austin, TX).
Facialis artery vasodilation studies. Facialis arteries (180 to 250 \( \mu \)m) were removed under deep anesthesia, cannulated, and connected to appropriate buffers for vasodilation experiments as previously described (31, 43). Vessels were preconstricted with the thromboxane A2 agonist U-46619 (10\(^{-8}\) to 10\(^{-6}\) mol/l), and the vasodilation that occurred in response to acetylcholine (Ach, 10\(^{-7}\) to 10\(^{-4}\) mol/l) in the presence and absence of \(N^\omega\)-nitro-l-arginine methyl ester (l-NAME), 100 \( \mu \)mol/l) was recorded (16, 43).

Statistical analysis. For in vitro functional analyses, statistical significance was determined using a paired \(t\)-test (Microsoft Excel, Microsoft, Redmond, WA). For in vivo experiments, unpaired \(t\)-tests were used for data that were distributed normally; Welch’s correction was applied when comparing populations with unequal SDs. For data that did not pass tests for normality, nonparametric Mann-Whitney \(U\)-tests were used for comparisons (GraphPad InStat 3, GraphPad, La Jolla, CA). Statistical comparison of vessel dilation in the absence and presence of l-NAME was done using two-way ANOVA (GraphPad Prism 5).

RESULTS

Peptide development. We combined the methods of phage display and ELISA to identify peptides that recognize and bind human Hb. From these experiments, we identified the amino acid sequence CHNLPTPWCA as our lead Hb-binding peptide and termed this peptide Hb-B10. Using biolayer interferometry (Octet RED96 System), we determined the binding affinity of Hb-B10 for oxyHb to be 21 ± 4 \( \mu \)mol/l (mean ± SD).

In vitro functional analysis. OxyHb is an effective scavenger of NO with a rate constant of 4.5 \( \times \) 10\(^{7}\) M\(^{-1}\) s\(^{-1}\) (15). Plasma from individuals with SCD consumes NO due to the elevated levels of oxyCF-Hb (49). To determine whether Hb-B10 inhibits NO scavenging by plasma with elevated CF-Hb, human SCD plasma was incubated with biotinylated Hb-B10 and streptavidin-coated magnetic beads followed by the removal of magnetic beads and associated Hb-B10/CF-Hb. Consistent with previous findings (58), control SCD plasma incubated with streptavidin-coated magnetic beads alone decreased the chemiluminescent signal, indicative of NO consumption. However, the addition of Hb-B10-coated magnetic beads to SCD plasma attenuated NO consumption (Fig. 1). These data demonstrate that Hb-B10 binds and efficiently promotes the removal of a factor in SCD plasma, which contributes to NO consumption. Since we have shown that Hb-B10 specifically binds CF-Hb and oxyHb is known to consume NO (15), these data indicate that Hb-B10 effectively removes CF-Hb from plasma in vitro.

In vivo clearance and tissue distribution of hE-Hb-B10. For Hb-B10 to decrease CF-Hb in the plasma in vivo requires that Hb-B10 both bind to and clear CF-Hb from the circulation. To accomplish both functions in vivo, Hb-B10 was coupled to a fragment of ApoE (hE, LRKLKRLRLR, residues 141–150), which has been shown to effectively clear lipoproteins from the circulation when linked to 18A, a well-characterized class A amphipathic helix that binds lipoproteins (13, 14, 17, 20).

To determine the peptide clearance rate, C57BL/6J mice were injected with FAM-labeled hE-Hb-B10, and fluorescence within the plasma was measured. The pharmacokinetic data from this study fitted a double-exponential equation, suggesting that hE-Hb-B10 is cleared from the plasma in two phases: a rapid phase with a \(t_{1/2}\) of 15.62 min and a slow phase with a \(t_{1/2}\) of 9.96 h (Fig. 2A). These clearance data are similar to those reported in previous studies (17, 20), where the hE peptide was linked to 18A to form the lipoprotein-clearing peptide hE-18A. Additionally, it has been previously demonstrated that peptides containing the hE sequence traffic to the liver (40). Consistent with these findings, FAM-labeled hE-Hb-B10 was detected in the livers of treated mice by fluorescent histological examination (Fig. 2, B and C). The FAM-labeled peptide was most prominent in perivascular hepatic cells at 1 h (Fig. 2, B and C, white arrows). Diffuse fluorescence within the liver at the 30-min and 4-h time points likely reflects the two phases of hE-Hb-B10 clearance (Fig. 2B, white arrows). Fluorescence in other tissues (the aorta, brain, lung, kidney, and spleen) was indistinguishable from background fluorescence. This suggests that hE-Hb-B10 likely traffics CF-Hb to the liver for clearance, similar to other hE-linked peptides (17, 40).

Effect of hE-Hb-B10 on CF-Hb in a murine model of acute hemolysis. To assess whether hE-Hb-B10 clears CF-Hb from the circulation in vivo, mice were treated with PHZ to induce acute hemolysis (32). As expected, PHZ increased circulating CF-Hb in the plasma of these mice (Fig. 3). Administration of hE-Hb-B10 at 10 and 21 h after PHZ treatment markedly reduced CF-Hb to levels that were comparable with plasma CF-Hb in vehicle-treated (nonhemolyzed) mice (Fig. 3).

Effects of hE-Hb-B10 in murine models of chronic hemolysis. Next, we investigated whether hE-Hb-B10 reduced CF-Hb in two murine models of chronic hemolysis: SS mice and HS mice (16, 44). Similar to previous studies (16, 35), PBS-treated SS and HS mice had increased concentrations of CF-Hb compared with AA mice at 1 h (Fig. 4, A and B, white arrows). The higher hemolytic rate in HS mice was reflected in the elevated reticulocyte count compared with SS mice (see Table 1). Importantly, hE-Hb-B10 reduced CF-Hb in the plasma of both SS and HS mice after 3 wk of daily treatment (Fig. 4, A and B). Together, these results suggest that hE-Hb-B10 effectively reduces CF-Hb in murine models of both acute and chronic hemolysis.

The reduction in CF-Hb in the above experiments could be the result of either increased clearance of CF-Hb or an overall

Fig. 1. Hb-B10 inhibits the nitric oxide (NO) scavenging capacity of plasma. The amount of NO consumed by plasma from individuals with sickle cell disease (SCD) was measured after an incubation in the absence [control (Ctrl)] or presence of 30 \( \mu \)M Hb-B10. Data are means ± SE; \( n = 4. \text{*} P < 0.05. \)
reduction in hemolysis. To determine if hE-Hb-B10 altered the hemolytic rate in chronic hemolysis models, we measured the complete blood count and reticulocyte count in vehicle- and peptide-treated mice. The stable total Hb level and reticulocyte count in both SS and HS mice suggest there were no overall changes in steady-state red blood cell destruction and the compensatory bone marrow response in treated mice (Table 1). These data provide strong support for the conclusion that hE-Hb-B10 reduces CF-Hb in vivo by promoting CF-Hb clearance from the circulation, rather than by reducing hemolytic rate. In addition, hE-Hb-B10 had no effect on total plasma LDH activity in either SS or HS mice (Table 1). As plasma LDH activity increases in response to both hemolysis and tissue damage (12), the high hemolytic rates in both models may limit our ability to detect subtle changes in LDH activity that might occur as a result of changes in tissue injury.

To ensure that the clearance of CF-Hb in our chronic hemolysis models via the liver did not result in liver toxicity, we measured plasma ALT activity in mice treated with PBS or hE-Hb-B10 (2, 46). Both SS and HS mice are known to have baseline chronic liver injury (2, 16, 35, 44, 56), which is reflected by elevated ALT activity in the plasma of both SS and HS mice compared with activity levels in their respective controls (Fig. 5, A and B). It is important to note that treatment with hE-Hb-B10 did not cause further liver toxicity as measured by plasma ALT activity. In fact, hE-Hb-B10 treatment actually lowered plasma ALT activity in both SS and HS mice (Fig. 5, A and B). These data are consistent with the notion that the reduction of CF-Hb by hE-Hb-B10 concomitantly decreased liver injury in these murine models of chronic hemolytic disease.

Effect of hE-Hb-B10 on nitric-oxide dependent vascular function. Facialis artery dilation in response to acetylcholine is NO-dependent in normal mice (43) and is attenuated in both SS and HS mice relative to respective control mice (Fig. 6, A and C) (16, 43). We found that the overall acetylcholine-induced facialis artery dilation is not improved in hE-Hb-B10-treated compared to PBS-treated SS or HS mice (Fig. 6, open diamonds vs. open circles). Interestingly, the acetylcholine-mediated vasodilation was inhibited by L-NAME (Fig. 6B), an
hE-Hb-B10 decreases cell-free Hb (CF-Hb) in a mouse model of acute hemolysis. Plasma CF-Hb levels were measured in C57BL/6 mice treated intraperitoneally with PBS, phenylhydrazine (PHZ; 4 μg/mouse), or PHZ followed by hE-Hb-B10 (PHZ + B10). Data are means ± SE. *P < 0.0001, PBS vs. PHZ; #P = 0.008, PHZ vs. PHZ + B10.

**DISCUSSION**

The direct role of CF-Hb in the pathobiology of hemolytic disease, particularly in SCD, is both poorly understood and controversial (9, 18), in part due to the lack of tools that directly target CF-Hb in the setting of active hemolysis. We have developed a novel Hb-binding peptide to further address this issue: Hb-B10. During peptide synthesis, Hb-B10 was covalently linked to a small fragment of ApoE (hE, residues 141–150) that has been shown to facilitate both the uptake of lipoproteins by small peptides and drug transport by liposomes via endocytic clearance through the ubiquitous heparan sulfate proteoglycan-associated pathway (14, 47, 51). Although modification of any small peptide might affect function, we found that the pharmacokinetic profile and liver localization of hE-Hb-B10 were very similar to previous reports for other hE-conjugated peptides (17, 20, 40).

Peptide hE-Hb-B10 is able to effectively attenuate the acute increase in CF-Hb concentrations in the PHZ-treated mice to nearly basal levels within 24 h. This rapid clearance of CF-Hb in PHZ-treated mice via hE-Hb-B10 is similar to the rapid reduction of plasma cholesterol observed in animals treated with hE-18A (17, 20). In addition, both hE-Hb-B10 and hE-18A are taken up by the liver, and the plasma clearance kinetics of both peptides are similar (17, 40). These data imply a common uptake and clearance mechanism for hE18A and hE-Hb-B10 via the heparan-sulfate proteoglycan-associated pathway of the liver (17, 20).

Importantly, hE-Hb-B10 effectively decreases CF-Hb in two distinct murine models of chronic hemolysis with strikingly different hemolytic rates (refer to Table 1). Furthermore, even though Hb-B10 was selected for optimal binding to normal human Hb, hE-Hb-B10 effectively promoted the clearance of human sickle Hb (SS mice) and mouse Hb (PHZ and HS mice) (16, 44). These data suggest that hE-Hb-B10 may be very useful for examining the pathology of CF-Hb in a variety of animal models and potentially human subjects with hemolytic diseases of varying severities.

**Table 1. Effect of hE-Hb-B10 on the hemolytic rate in SS and HS mice**

<table>
<thead>
<tr>
<th></th>
<th>Whole Blood Hb, g/dl</th>
<th>Reticulocyte Count, percentage of red blood cells</th>
<th>Plasma Lactate Dehydrogenase Activity, IU/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>SS mice</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>7.0 ± 0.7 (n = 9)</td>
<td>53 ± 14 (n = 9)</td>
<td>211 ± 48 (n = 9)</td>
</tr>
<tr>
<td>hE-Hb-B10</td>
<td>6.3 ± 0.9 (n = 7)</td>
<td>55 ± 12 (n = 7)</td>
<td>173 ± 42 (n = 7)</td>
</tr>
<tr>
<td>HS mice</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>5.3 ± 0.7 (n = 14)</td>
<td>94 ± 3 (n = 7)</td>
<td>860 ± 173 (n = 13)</td>
</tr>
<tr>
<td>hE-Hb-B10</td>
<td>4.7 ± 0.8 (n = 11)</td>
<td>93 ± 4 (n = 11)</td>
<td>963 ± 256 (n = 12)</td>
</tr>
</tbody>
</table>

Values are means ± SD; n, no. of mice/group. SS mice, mice with sickle cell disease; HS mice, mice with hereditary spherocytosis.

**Fig. 4.** hE-Hb-B10 decreases CF-Hb in mouse models of chronic hemolysis. A: plasma CF-Hb levels in AA and SS mice were measured after daily intraperitoneal treatment with PBS or hE-Hb-B10 for 3 wk. Data are means ± SE. *P < 0.0001, control mice (AA and Ctrl mice) compared with their respective mutant mice (SS or HS mice); #P = 0.003, PBS-treated compared with hE-Hb-B10-treated mutant mice.

**Fig. 3.** hE-Hb-B10 decreases cell-free Hb (CF-Hb) in a mouse model of acute hemolysis. Plasma CF-Hb levels were measured in C57BL/6 mice treated intraperitoneally with PBS, phenylhydrazine (PHZ; 4 μg/mouse), or PHZ followed by hE-Hb-B10 (PHZ + B10). Data are means ± SE. *P < 0.0001, PBS vs. PHZ; #P = 0.008, PHZ vs. PHZ + B10.
SS and HS mice have baseline chronic liver injury indicated by histopathology and elevated plasma ALT activity (2, 16, 35, 44, 56). Thus potential hepatotoxicity of hE-Hb-B10 in the chronic hemolysis models is of concern. Therefore, we measured plasma ALT activity to ensure that the transport of CF-Hb by hE-Hb-B10 did not potentiate liver injury in either model. In fact, we found that hE-Hb-B10 actually attenuated plasma ALT activity in both murine models. Thus reducing circulating levels of CF-Hb with hE-Hb-B10 appears to improve markers of liver injury in the SS and HS mice.

![Graph A](image1)

**Fig. 5.** hE-Hb-B10 reduces plasma alanine aminotransferase (ALT) activity in mouse models of chronic hemolytic anemia. **A:** Plasma ALT activity in AA and SS mice was measured after daily intraperitoneal treatment with PBS or hE-Hb-B10 for 3 wk. **B:** Plasma ALT activity in Ctrl and HS mice was measured after daily intraperitoneal treatment with PBS or hE-Hb-B10 for 3 wk. Data are means ± SE. *P = 0.003, AA mice compared with SS mice, and P < 0.0001, Ctrl mice compared with HS mice; #P ≤ 0.02, PBS-treated compared with B10-treated mutant mice.

![Graph B](image2)

**Fig. 6.** hE-Hb-B10 restores N^o^-nitro-L-arginine methyl ester (L-NAME) inhibition of vasodilation in response to ACh in SS mice. Facialis arteries (180–250 μm) were isolated from SS and HS mice after daily intraperitoneal treatment with PBS or hE-Hb-B10 for 3 wk. Vasodilation in response to ACh was measured in the absence or presence of the NO synthase inhibitor L-NAME. **A:** Comparison of vasodilation of arteries from representative AA mice versus SS mice treated with PBS in the absence and presence of L-NAME. **B:** Comparison of vasodilation of arteries from SS mice treated with hE-Hb-B10 in the absence or presence of L-NAME. **C:** Comparison of vasodilation of arteries from representative Ctrl mice versus HS mice treated with PBS in the absence and presence of L-NAME. **D:** Comparison of vasodilation of arteries from HS mice treated with hE-Hb-B10 in the absence or presence of L-NAME. D_max, maximum diameter. Data are means ± SD; n = 9 vessels from 5 mice for SS mice treated with PBS with or without L-NAME, 9 vessels from 6 mice for SS mice treated with hE-Hb-B10 with or without L-NAME, 13 vessels from 9 mice for HS mice treated with PBS, 10 vessels from 6 mice for HS mice treated with PBS with L-NAME, 12 vessels from 9 mice for HS mice treated with hE-Hb-B10, and 8 vessels from 5 mice for HS mice treated with hE-Hb-B10 with L-NAME. *P < 0.05, vessel dilation without L-NAME compared with vessel dilation with L-NAME. Representative data from AA mice (5 vessels from 3 mice) and Ctrl mice (7 vessels from 4 mice) are for comparison only and were not included in the statistical analyses.

![Graph C](image3)

![Graph D](image4)
that hE-Hb-B10 treatment decreased plasma ALT activity in both SS and HS mice suggests that, in addition to not inducing liver injury, hE-Hb-B10 treatment may actually improve baseline liver function through efficient clearance of CF-Hb. It is possible that CF-Hb directly contributes to liver injury by inducing oxidative tissue damage through lipid oxidation (5, 36, 48). Thus the clearance of CF-Hb could lessen liver oxidative injury. In addition, CF-Hb inhibits the protective mechanisms of NO by rapidly reacting with this molecule and decreasing its bioavailability (23, 49, 50). Therefore, lowering CF-Hb with hE-Hb-B10 could inhibit these direct effects of CF-Hb. Another explanation for the decrease in ALT in hE-Hb-B10-treated mice is that increased cellular uptake of hemoglobin/heme/iron may induce protective/anti-inflammatory mechanisms in the liver. For example, the cytoprotective enzyme heme oxygenase-1 (HO-1) is induced by its substrate (heme) (34, 54). Others have demonstrated that potentiation of HO-1 expression or administration of HO-1 enzymatic products is protective in SS mice (4, 6, 7). Further studies are required to elucidate the mechanisms by which hE-Hb-B10 reduces liver injury as well as to identify any additional potential therapeutic effects of this peptide for treatment of hemolytic anemia.

It is interesting that plasma ALT activity is higher in SS mice compared to HS mice. These differences develop despite of the fact that SS mice have a much lower hemolytic rate than HS mice. Even though hemolysis and heme-induced oxidative injury likely contribute to liver damage in both mouse models, the marked increase in plasma ALT in the SS mice may result from tissue injury induced by vascular occlusion because of erythrocyte sickling. Another divergence of the two hemolytic murine models used in this study is the vasodilatory response of the facialis artery to acetylcholine in the presence of the NOS inhibitor l-NAME. Vessels from HS mice still appear to rely on NO production for vasodilation, albeit attenuated compared with Ctrl mice. In contrast, vessels from SS mice still have a measurable vasodilation that occurs in the presence of l-NAME. Thus vasoocclusion due to erythrocyte sickling may also contribute to vessel injury in SS mice, requiring compensatory pathways to maintain some amount of vascular tone. Potential mediators of facialis artery vasodilation in SS mice include the cyclooxygenase 2 or HO-1 pathways (29), as well as endothelial-independent vasodilation by cAMP (23). The finding that hE-Hb-B10 returns vessels from SS mice to a more NO-dependent phenotype suggests hemoglobin/heme trafficking to the liver induces cytoprotective mechanisms that improve vascular function. Belcher et al. (7) have shown that potentiating the expression of HO-1 in the liver of SS mice improved vascular stasis in areas distal to the liver (dorsal skin folds). It is also possible that decreasing CF-Hb improved NO bioavailability, resulting in NO-mediated cytoprotection to combat occlusion-induced vessel injury. Future studies are needed to define the mechanism(s) by which hE-Hb-B10 alters vessel function in SS mice.

In summary, these data demonstrate that hE-Hb-B10 is a novel and effective tool to investigate fundamental questions about the role(s) of CF-Hb in the complex pathobiology of vascular and organ injury in hemolytic anemia. These findings also indicate that hE-Hb-B10 may have therapeutic benefit by suppressing Hb-mediated tissue injury. Future studies are required to determine both the mechanisms of hE-Hb-B10-mediated CF-Hb clearance by the liver as well as the associated reductions in plasma ALT activity and alterations in vessel function.

ACKNOWLEDGMENTS

hE-Hb-B10 was designed through the efforts of Translational Vascular Biology Program. The authors greatly appreciate the technical assistance of Weiling Wang, Thomas Foster, and Trudy Holyst.

GRANTS

This work was supported by American Heart Association Grant (5030073N (to N.J. Wandersee), National Institutes of Health Grants HL-071214 (to K.A. Pritchard, Jr.), HL-081139 (to K. A. Pritchard, Jr., and C.A. Hillery), HL-102856 (to K. A. Pritchard, Jr., and C.A. Hillery), HL-090903 (to N. Hogg, C. A. Hillery, and N. J. Wandersee), NS-070711 (to C.A. Hillery and N.J. Wandersee), 5-T32-HL-007209-33 (to M.S. Hansen), 5-F31-HL-092773 (to T.C. Flewelen), and EB-001980 (to the National Biomedical Electron Paramagnetic Resonance Center), the Department of Pediatric Surgery (to H. Xu), and the Midwest Athletes Against Childhood Cancer Fund (to N.J. Wandersee).

DISCLOSURES

C. A. Hillery is a consultant for Bayer Pharmaceuticals.

AUTHOR CONTRIBUTIONS


REFERENCES


