quantities sufficient for clinical trials. Sterilization of the hemoglobin, prior to encapsulation, is performed using heat, and antioxidants are co-encapsulated to retard hemoglobin oxidation. Oxygen affinity is regulated to any desired P50 by co-encapsulation of allosteric effectors, and this group has contributed important studies on the effect of different P50 on oxygen delivery to tissues by HbV. The product is claimed to be stable when stored for up to 2 years.

A commercial effort has been launched in Japan, and it is hoped that HbV could be in human clinical trials within the next few years.

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Albumin-Heme: A Synthetic Heme-Based Oxygen Carrier

Chapter 46

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INTRODUCTION

The risk of transmission of viral illness by transfused blood has become extremely low and the transfusion of donor blood is currently a routine procedure. However, this level of safety has been achieved at great cost, and hepatitis virus or unknown pathogens cannot be completely excluded by the NAT system. Furthermore, the transfusion of donor blood requires cross-matching and compatibility tests to avoid a hemolytic reaction in the recipient, and the purified red blood cells (RBC) need to be stored in the refrigerator at 4°C. These requirements limit the availability of blood in a disaster or emergency. Against this background, several types of hemoglobin (Hb)-based oxygen carriers (HBOCs) have been studied as potential RBC substitutes or O_2 therapeutic reagents (Chang, 1997; Tsuchida, 1998; Winslow, 1999; Squires, 2002; Greenburg and Kim, 2004). Unfortunately, these materials do not fulfill all the requirements of blood replacement compositions. The first concern is the source of human Hb, which is limited by the availability of outdated human blood. Animal blood raises concerns about the transmission of animal pathogens, as Hb products potentially carry risks due to the biological origin of the raw materials. The second problem of the HBOCs (i.e., modified Hb) is the high colloid osmotic pressure (Keipert and Chang, 1988) and its vasoconstriction effect (Schultz *et al.*, 1993; Abassi *et al.*, 1997; Moisan *et al.*, 1998). About 50 per cent of the products in advanced clinical trials still increase blood pressure and decrease cardiac output (Squires, 2002). The precise mechanism of this hypertension is controversial, but many researchers suspect that the Hb molecules penetrate the vascular endothelium and capture the endothelial-derived relaxing factor (EDRF), namely NO. Others believe that the excessive delivery of oxygen to arteriolar vascular walls induces autoregulatory vasoconstriction (Guyton *et al.*, 1964; Tsai *et al.*, 1995; Rohlfs *et al.*, 1998; Winslow, 2000).

RATIONALE FOR ALBUMIN-HEME

In our circulatory system, free hemin (iron(III) complex of protoporphyrin IX dissociated from metHb) is captured by hemopexin, which is a unique protein having an extremely high binding constant of hemin ($K > 10^{12} M^{-1}$) (Tolosano and Altruda, 2002). Crystal structure analysis of the hemopexin–hemin complex has revealed that the hemin is tightly bound by double histidine

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coordinations to the central ferric ion and multiple hydrogen bondings with the amino acid residue (Paoli *et al.*, 1999). Nevertheless, the concentration of hemopexin in the plasma is rather low (<17 μM) and human serum albumin (HSA) may provide a reserve binding capacity of hemin in various conditions, for instance trauma, inflammation, hemolysis etc. In fact, HSA binds hemin with a relatively high affinity ($K > 10^8 M^{-1}$) (Adams and Berman, 1980). If HSA can transport O_2 like Hb, it would be of extreme medical importance not only as a blood replacement composition but also as an O_2 therapeutic reagent.

We have found that a series of super-structured heme derivatives with a covalently linked proximal-base were incorporated into HSA, and the obtained red-colored albumin–heme hybrids (Figure 46.1) can reversibly bind and release O_2 under physiological conditions in the same manner as Hb and myoglobin (Mb) (Komatsu *et al.*, 1999, 2000, 2001a, 2002; Tsuchida *et al.*, 1999; Nakagawa *et al.*, 2004). Since recombinant HSA (rHSA) is manufactured on a large scale by yeast expression, the rHSA–heme hybrid has become entirely synthetic hemoprotein and absolutely free of infectious pathogens. Our recent animal experiments demonstrated that rHSA-heme actually works as an oxygen-carrying plasma protein in the bloodstream (Tsuchida *et al.*, 2000; Komatsu *et al.*, 2004). Although the NO-binding affinity of rHSA-heme is higher than that of Hb (Komatsu *et al.*, 2001b), it does not induce an unfavorable vasopressor effect at all

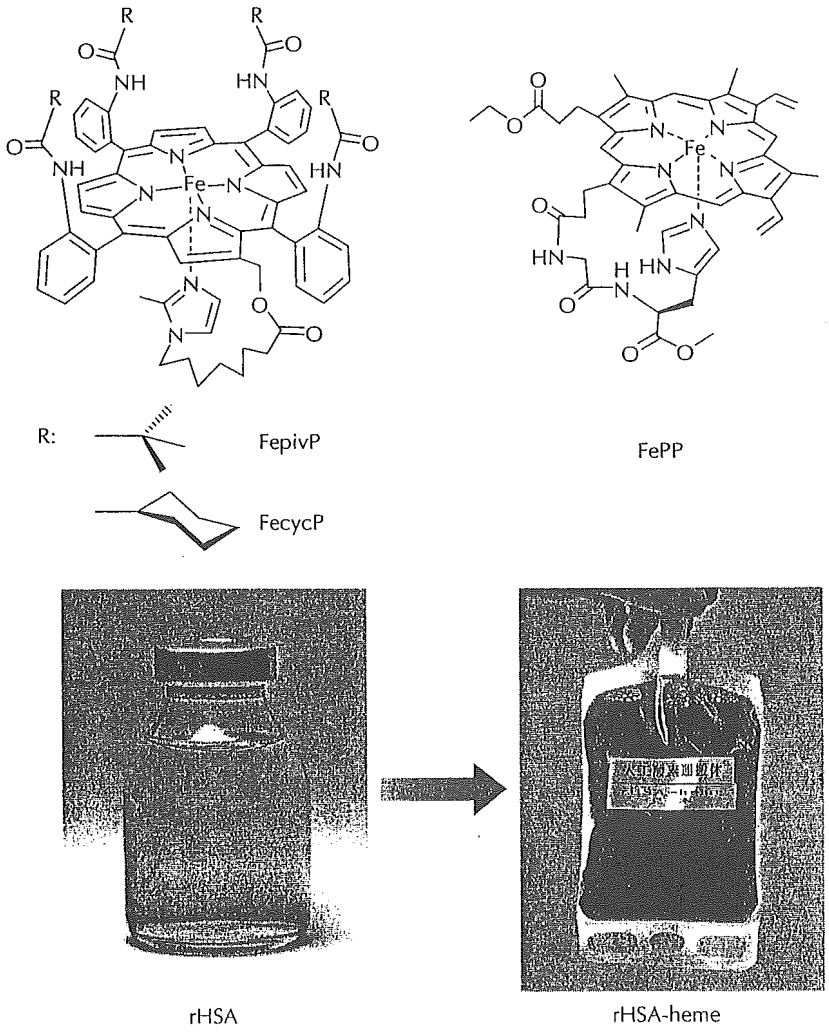

Figure 46.1 Super-structured heme derivatives for the albumin-heme hybrids and the red-colored rHSA-heme solution ([rHSA] = 5 g/dl). See color plate 23.

(Tuschida *et al.*, 2003). We suspect that the electrostatic repulsion between the albumin surface and glomerular basement membrane around the endothelial cell retards the rapid leakage of the rHSA-heme molecule and quick scavenging of NO. The albumin-heme is now recognized to be a promising material for a new class of RBC substitutes. In this chapter, we describe the O_2-transporting efficacy and preclinical safety of this synthetic heme-based O_2-carrier.

OXYGEN BINDING AND PHYSICOCHEMICAL CHARACTERISTICS

From the 30 super-structured heme compounds that were all synthesized by the authors, we found that oxygenated rHSA-FecycP showed a high stability against the autoxidation; its half-time against the ferric form *in vitro* (9 h at 37°C) was close to that of the native Mb (Komatsu *et al.*, 2002). We selected rHSA-FecycP with a similar *P*50 value (34 mmHg at 37°C) to that of RBCs as the most suitable material for an artificial O_2-carrier. The physicochemical characteristics and shelf-life of the rHSA-heme solution ([rHSA], 5 g/dl; heme/rHSA, 4 (mol/mol); isoelectric point, 4.8; COP, 18 mmHg; viscosity, 1.1–1.2 cPs; shelf-life greater than 2 years) had already been reported elsewhere (Komatsu *et al.*, 1999, 2002; Tsuchida *et al.*, 2002).

BLOOD COMPATIBILITY *IN VITRO*

The viscosity of the rHSA-heme solution (1.2 cPs at a high shear rate of 230 s^{-1}) was much lower than that of whole blood (4.0 cPs) and exhibited Newtonian type shear rate dependence just like rHSA itself. After mixing the rHSA-heme solution into whole blood at 10–44 per cent of the volume, the heme concentration in the plasma phase remained constant for 6 hours at 37°C, and no significant time dependence was observed in the numbers of RBCs, white blood cells and platelets (Huang *et al.*, 2003). The microscopic observations clearly showed that the shapes of the RBC had not been deformed during the measurement period. These results suggest that the rHSA-heme has no effect on the morphology of the blood cell components *in vitro*. With respect to the blood coagulation parameters (prothrombin time and activated partial thromboplastin time), the coexistence of rHSA-heme has only a negligibly small influence. Moreover, it was also shown that the rHSA-heme solution has no influence on the complement factors (CH50, SC5b-9) and platelet activation. Although more functional assay is necessary to establish firmly the biocompatibility of rHSA-heme with whole blood, we can conclude that it has a good compatibility with blood cells.

IN VIVO EFFECTS

Blood pressure effects

The administration of extracellular HBOCs often elicits an acute increase in blood pressure by vasoconstriction. At the beginning of this study, our concern was that the small rHSA-heme molecules (8 × 3 nm) injected into the blood vessels would be eliminated from the circulation, and contribute to the significant consumption of NO in the interstitial space between the endothelium and vascular smooth muscle. In fact, rHSA-heme strongly binds NO; the NO-binding affinity ($P50^{NO} = 1.8 \times 10^{-8}$ mmHg) is nine-fold higher than that of the Hb, and is high enough to react with 1 μM NO in the wall of the vasculator (Komatsu *et al.*, 2001b). In order to clarify the hemodynamic behavior after the administration of this entirely synthetic O_2-carrying hemoprotein, we tested a top-load dose of the rHSA-heme solution in anesthetized rats (Tsuchida *et al.*, 2003). Contrary to our expectations, only a negligibly small change in the mean arterial pressure (MAP) was observed after the administration of the rHSA-heme solution (5 g/dl, 300 mg/kg; Figure 46.2a). If anything, the difference from the baseline (ΔMAP) slowly decreased to −6.8 ± 3.4 mmHg within 20 minutes and remained constant during the monitoring period. The response was completely the same as that observed following infusion with an equivalent volume of rHSA (5 g/dl) in this experimental set-up. In contrast, the administration of extracellular Hb solution elicited an acute increase in blood pressure (ΔMAP 16 ± 1.9 mmHg), which followed a graduated decrease throughout the 60-minute period of observation (Tsuchida *et al.*, 2003). Why does rHSA-heme not induce the hypertension? The answer probably lies in the negatively charged molecular surface of the albumin vehicle. One of the unique characteristics of serum albumin is its low permeability through the muscle capillary

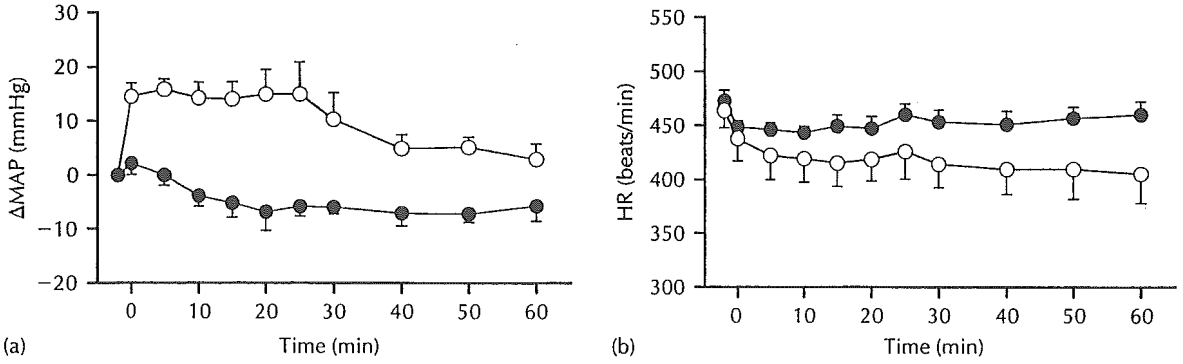

Figure 46.2 Changes of (a) MAP and (b) HR in anesthetized rats before and after infusion of rHSA-heme solution (n = 5) (●, rHSA-heme group; ○, Hb group). MAP is represented as change from the basal value (ΔMAP) just before the infusion with mean ± SEM (n = 5) (basal value is 90.1 ± 3.0 mmHg). HR was shown as mean ± SEM (n = 5) (Tsuchida, 2003).

pore, which is less than 1/100 that for Hb due to the electrostatic repulsion between the albumin surface and the glomerular basement membrane around the endothelial cells. In the blood vessels, rHSA-heme presumably circulates for a longer time compared to Hb without extravasation. The heart rate (HR) responses after the rHSA-heme injection were also negligibly small (Figure 46.2b). Visualization of the intestinal microcirculatory changes clearly showed that the widths of the venule and arteriole are fairly constant (Tsuchida *et al.*, 2003).

Hemodilution

The physiological responses to a 30 per cent exchange transfusion with rHSA-heme solution after 70 per cent hemodilution with 5 g/dl rHSA were investigated using anesthetized rats (Komatsu *et al.*, 2004). First, the isovolemic 70 per cent hemodilution was carried out using 5-g/dl rHSA solution. The blood withdrawal via the common carotid artery (2 ml) and the rHSA infusion from the femoral vein (2 ml) (each 1 ml/min) were repeated for nine cycles until Hct was reduced to 13.6 per cent (32 per cent of the initial Hct value of 42.6 per cent). After 10 minutes, a 30 per cent volume of the circulatory blood was withdrawn, producing a severe hemorrhagic shock state. The same volume of the samples was then intravenously injected. As negative or positive control groups, the rats were infused with the 5-g/dl rHSA solution (rHSA group) or the shed rat blood ([heme] = 5.3 mM, whole blood group). The circulation parameters, blood parameters, renal cortical PO_2 (PtO_2(R)) and muscle tissue PO_2 (PtO_2(M)) were carefully monitored for 60 minutes after the injection.

Following administration of the 5-g/dl rHSA solution, the MAP, HR, respiration rate, PtO_2(R), PtO_2(M), arterial blood O_2 pressure (PaO_2), venous blood O_2 pressure (PvO_2), and arterial blood CO_2-pressure ($PaCO_2$) did not recover, leading to death within 32 minutes (Figure 46.3). By contrast, the infusion of whole blood improved these values, except for PtO_2(M), to their initial levels. In the rHSA-heme group, the animals survived over 60 minutes after the infusion, and the HR, respiration rate, PtO_2(R) and PvO_2 showed similar recoveries to those as observed in the whole blood group (Komatsu *et al.*, 2004). MAP, PtO_2(M), PaO_2, pH and PCO_2 also significantly recovered. We are certain that the albumin-heme solution has the potential to resuscitate hemorrhagic shock, stabilize the blood circulation, and transport oxygen throughout the body.

PRECLINICAL SAFETY

In order to evaluate the preclinical safety of this synthetic O_2 carrier, we performed a 20 per cent exchange transfusion with rHSA-heme into anesthetized rats and measured the time courses of the circulation parameters (MAP, HR, respiration rate) and blood parameters (PaO_2, PvO_2, pH, blood cell numbers) for 6 hours, which is adequate time to identify acute toxicity (Huang *et al.*, 2004a). After stabilization of the animals' condition, the

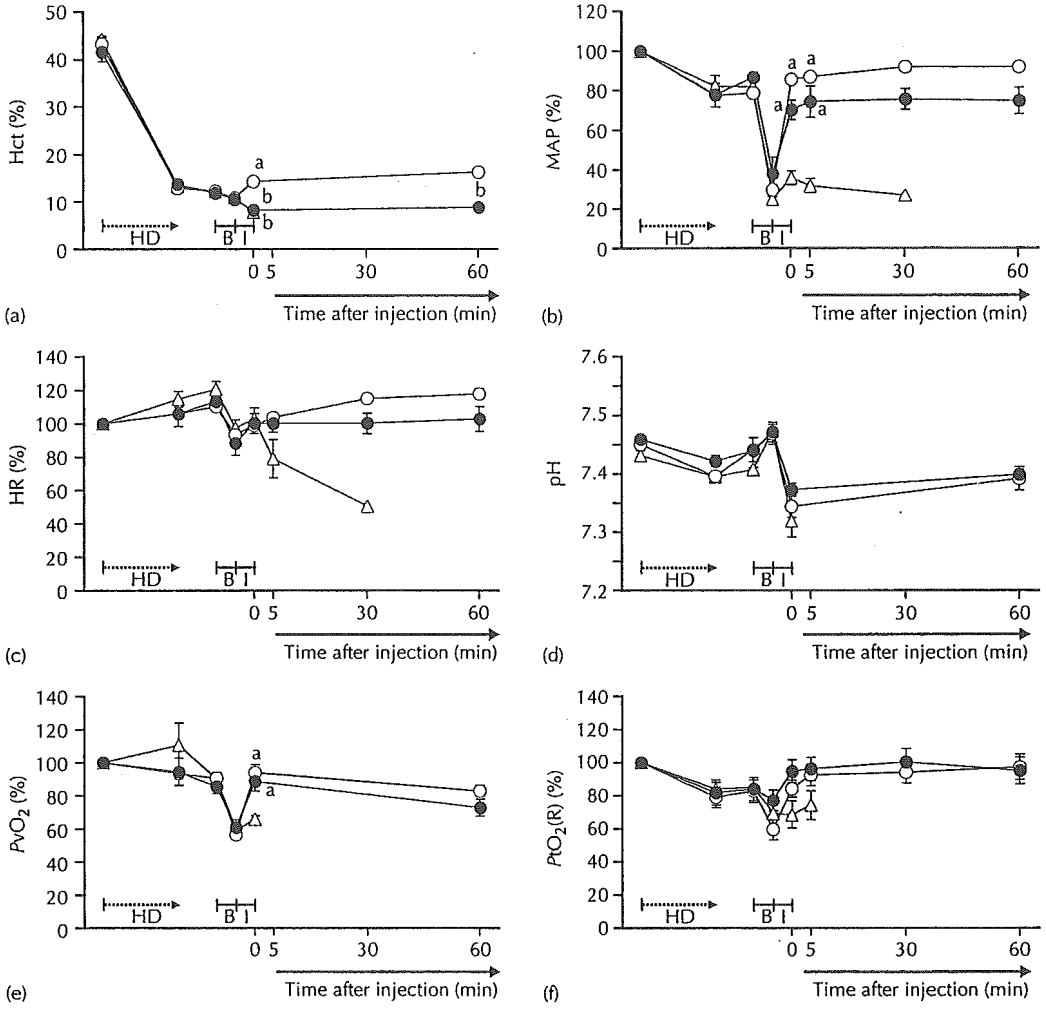

Figure 46.3 Time courses of (a) Hct, (b) MAP, (c) HR, (d) pH, (e) PvO_2 and (f) PtO_2(R) in anesthetized rats after 70 per cent hemodilution with rHSA and 30 per cent exchange transfusion with rHSA-heme solution (n = 6) (●, rHSA-heme group; ○, whole blood group; △, rHSA group). MAP, HR, PvO_2 and PtO_2(R) are represented as percentage ratios of the basal values with mean ± SEM. Hct, HR and pH were shown as mean ± SEM. HD, hemodilution; B, bleeding; I, sample injection. $^{a}P < 0.05$ versus rHSA group. $^{b}P < 0.05$ versus whole blood group (Komatsu, 2004).

20 per cent exchange transfusion was performed by 1 ml blood withdrawal via the common carotid artery and 1 ml rHSA-heme infusion from the femoral vein (each 1 ml/min) with four repeating cycles.

The appearance of all the animals showed absolutely no change for 6 hours after the exchange transfusion. The physiological responses of the blood circulation, gas equilibria and blood cell numbers in the rHSA-heme group were almost the same as those of the control group (surgery treatments without infusion) and rHSA groups (Figure 46.4; Huang *et al.*, 2004a). MAP and HR did remain constant after the injection of the rHSA-heme, suggesting again that the albumin-based O_2 carrier does not induce the vasoconstriction. It is also noteworthy that autoxidation of the ferrous rHSA-heme to the ferric state was retarded in the bloodstream; the half-time of the oxygenated rHSA-heme *in vivo* was approximately four-fold longer than that *in vitro* (Tsuchida *et al.*, 2000). It was found that autoxidated rHSA-hemin was certainly reduced in the whole blood suspension. A physiological concentration of ascorbic acid continuously provided by RBC probably re-reduces the ferric hemin, leading to the apparent long lifetime of the oxygenated species.

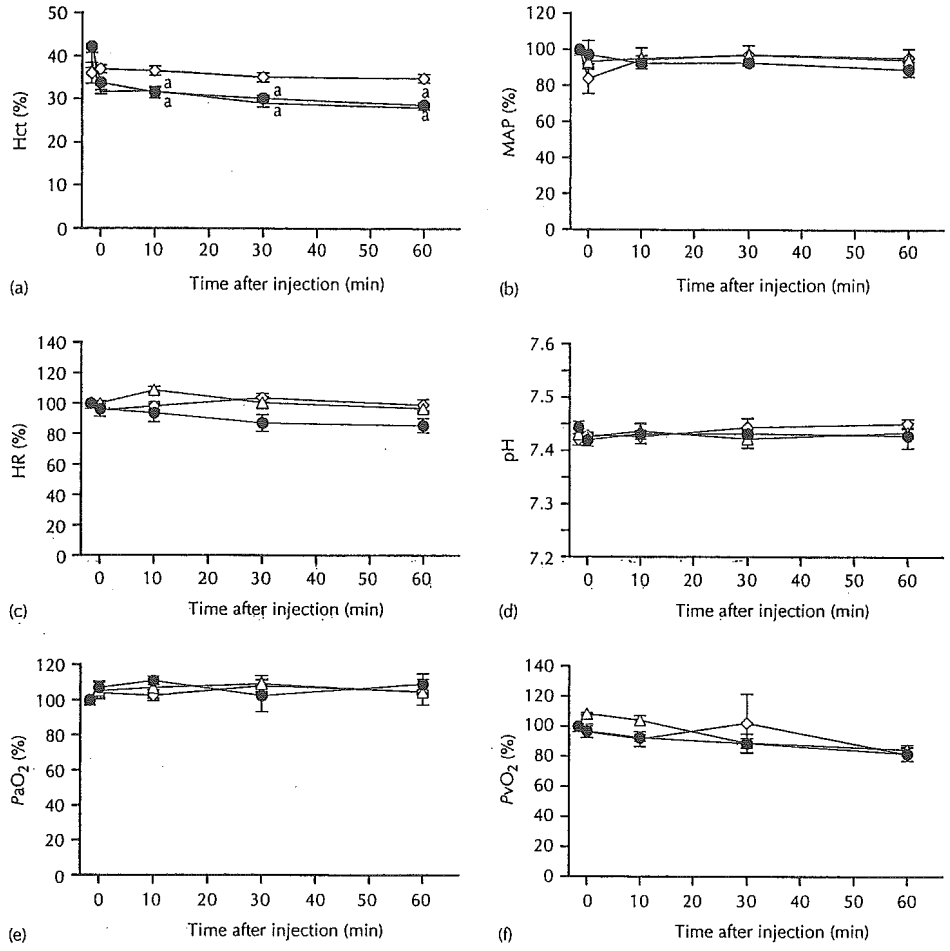

Figure 46.4 Time courses of (a) Hct, (b) MAP, (c) HR, (d) pH, (e) PaO_2 and (f) PvO_2 in anesthetized rats after 20 per cent exchange transfusion with rHSA-heme or rHSA solution (n = 6) (◇, control group (only surgery treatments without infusion); △, rHSA group; ■, rHSA-heme group). MAP, HR, PaO_2 and PvO_2 are represented as percentage ratios of the basal values with mean ± SEM. Hct, HR and pH are shown as mean ± SEM. (Huang, 2004a).

Furthermore, 20 per cent exchange transfusions with rHSA-heme into anesthetized rats were followed by blood biochemical tests of the withdrawn plasma and histopathology observations of the vital organs for 7 days (Huang *et al.*, 2004b).

In the albumin-heme group, a total of 30 analytes showed almost the same values, by blood biochemical tests, as those observed in the reference rHSA group, implying that there was no significant toxicity caused by the exchange transfusion with rHSA-heme (Huang *et al.*, 2004b). Histopathology observations implied that the administration of rHSA-heme did not produce any negative side effects on the vital organs. All these results showed the preclinical safety of the rHSA-heme solution.

FUTURE RESEARCH

As described in this chapter, results have shown O_2-transporting efficacy and initial clinical safety of the rHSA-heme solution that allow us to undergo further advanced preclinical testing of this synthetic O_2 carrying plasma protein. Exchange transfusion with rHSA-heme into beagles is now under investigation.

Furthermore, rHSA-heme as a monomolecular O_2 carrier was tested for its ability to increase O_2 tension in the hypoxia of the solid tumor rat model. By the direct administration of the rHSA-heme solution (10 ml/kg) into the ascites hepatoma LY80 tumor on the femur, the O_2 tension of the hypoxic region immediately increased

to 3.45 ± 1.43 mmHg, which corresponds to a 2.4-fold increase compared to that of the baseline value (Kobayashi *et al.*, 2003). These high O_2 levels continued for 300 s after the infusion. While more research is required to consider how rHSA-heme behaves in the tumor blood vessel and is related to the increase in the O_2 partial pressure, the present results obviously indicate that rHSA-heme led to increased O_2 release in the hypoxic region in the solid tumor. Experiments regarding combined treatment with the rHSA-heme administration and radiation therapy are currently underway.

ACKNOWLEDGMENTS

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EDITOR'S SUMMARY

Albumin-heme is a unique product – an oxygen transporter that is totally synthetic. By this approach, human serum albumin is produced in a recombinant yeast system, and then synthetic heme is coordinated to its surface. Up to 8 heme groups per molecule have been incorporated so far.

Albumin-heme has been prepared to have a P_{50} similar to that of red blood cells, but the oxygen binding is not cooperative. It avidly binds NO, but is not hypertensive in preliminary animal tests. It appears to be as stable with regard to oxidation as native human hemoglobin. A number of early preclinical tests have been performed, including biocompatibility and effects on coagulation, and no significant toxic effects have been noted.

While it is still early in the development of albumin-heme as a therapeutic agent for use in humans, and the cost of production is likely to be high, it is an intriguing product that could find use in specialized applications such as oxygenating tumors to increase the effects of radio- or chemotherapy.

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Effects of Hemoglobin Vesicles on Resting and Agonist-Stimulated Human Platelets In Vitro

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Abstract: Hemoglobin vesicles (HbV) are artificial oxygen carriers that encapsulate a concentrated hemoglobin (Hb) solution with a phospholipid bilayer membrane. The oxygen transporting ability of HbV in vivo has been demonstrated by the transfusion of HbV into hemorrhagic shock rodent models. However, the compatibility of HbV with human blood cells must be evaluated. Preincubation of platelets with concentrations of 20% or 40% HbV had no effect on the binding of PAC-1, a monoclonal antibody that detects activation-dependent conformational changes in $\alpha_{IIb}\beta_3$ on platelets, or the surface expression of CD62P in whole blood. ADP-induced increases in PAC-1 binding were significantly enhanced by exposing the platelets to concentrations of either 20% or 40% HbV, whereas the ADP-induced increases in CD62P expression were not affected by HbV treatment at either concentration. Preincubation of platelet-rich plasma (PRP) with HbV minimally reduced the spontaneous release of TXB_2 and RANTES, but did not significantly affect the formation of TXB_2 or the release of RANTES and β-TG in platelets stimulated with ADP. Similarly, preincubation of PRP with HbV minimally reduced the spontaneous release of RANTES but did not significantly affect the formation of TXB_2 or the release of RANTES and β-TG in platelets stimulated with collagen, although collagen-induced serotonin release tended to decrease with HbV pretreatment. These data suggest that the exposure of human platelets to high concentrations of HbV (up to 40%) in vitro did not cause platelet activation and did not adversely affect the formation and secretion of prothrombotic substances or proinflammatory substances triggered by platelet agonists, although one of the earliest events in ADP-induced platelet activation was slightly potentiated by HbV pretreatment at the doses tested. Taken together,

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these results imply that HbV, at concentrations of up to 40%, do not have any aberrant interactions with either unstimulated or agonist-induced platelets.

INTRODUCTION

Vigorous efforts have been made to develop hemoglobin (Hb)-based oxygen carriers (HBOCs) for use as red blood cell substitutes [1], and some of these carriers are now in the final stages of clinical trials [2–4]. HBOCs offer several potential benefits for red blood cell transfusion applications, including the absence of blood-type antigens and infectious viruses and the ability to be stably stored for long time periods [5]. HBOCs can be categorized into two types: acellular modified Hb molecules and cellular liposome-encapsulated Hb, or Hb vesicles (HbV) [6]. Acellular modified Hb molecules are composed of intramolecularly cross-linked Hb, recombinant cross-linked Hb, polymerized Hb, or intramolecularly polymer-conjugated Hb. An acellular polymerized bovine Hb has already been used in clinical practice in South Africa.

Cellular HbV have a phospholipid vesicle structure and contain concentrated Hb molecules, similar to actual red blood cells [7–11]. Although HbV have not been clinically tested, the oxygen transporting abilities of HbV have been shown to be sufficient using a 40% exchange transfusion with HbV suspended in saline [8] and a 90% exchange transfusion with HbV in the presence of albumin as a plasma expander in rats [7]. Surface modification of HbV with poly(ethyleneglycol)-phospatidylethanolamine reduced the viscosity by suppressing inter-vesicular aggregation, allowing prompt blood circulation in vivo [9]. A sufficient O_2 transporting ability, comparable with that of blood, was also established in another model [11], and the prompt metabolism of HbV in the reticulo-endothelial system has been demonstrated [10].

The biocompatibility of HbV is an important factor for the clinical use of these materials. The administration of HbV could lead to interactions with blood cells, including platelets. Circulating platelets bind to the subendothelial matrix of injured vessels and subsequently become activated, resulting in the release or the expression of components in their intracellular granules and the formation of metabolic products. These products include prothrombotic substances (e.g., adenine nucleotides, thromboxane A_2 [TXA_2], serotonin, and CD62P) [12] and an array of potent proinflammatory chemokines (e.g., RANTES, MIP-1) [13]. Prothrombotic substances function as agonists for the recruitment of additional platelets into the evolving thrombus. Chemokines released from the activated platelets trigger the recruitment of leukocytes into the evolving thrombus and play a large role in the initiation and perpetuation of inflammatory responses. Platelet activation is apparently necessary to prevent bleeding in vivo; however, nonphysiological activation leads to pathological thrombosis and the

modulation of inflammatory responses. With this in mind, the biocompatibility of HbV and human platelets was evaluated by examining the effect of HbV on CD62P expression and the binding of activation–dependent $\alpha_{IIb}\beta_3$ antibody PAC-1 to platelets in the presence or absence of agonists in vitro; these two markers are the most frequently used markers of platelet activation. We also studied the effects of HbV on the secretion of other substances (i.e., serotonin, RANTES, and β-thromboglobulin [β-TG]) and the formation of thromboxane B_2 (TXB_2), a metabolite of TXA_2.

MATERIALS AND METHODS

HbV

HbV suspended in phosphate buffered saline were prepared as previously described [14]. The encapsulated carbonylhemoglobin contained pyridoxal 5′-phosphate (PLP) at a molar ratio of [Hb]/[PLP] = 1/2.5 as an allosteric effector and 5 mM of DL-homocysteine. The lipid bilayer was composed of 1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine, cholesterol, 1,5-dipalmitoyl-L-glutamate-*N*-succinic acid, and polyethyleneglycol-1, 2-distearyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-[poly (ethylene glycol) (5,000)] at a molar ratio of 5:5:1:0.033. The Hb concentration of the HbV dispersion was adjusted to 10 g/dl. The HbV particle size was nearly 240 ± 60 nm in diameter.

Determination of CD62P and PAC-1 Expression by Flow Cytometry

The expression of CD62P and PAC-1 on platelets was measured as described previously, with slight modifications [15, 16]. Citrated whole blood was obtained from unselected healthy subjects. Whole blood (520 μl) was incubated with 480 μl of HbV or empty liposomes (at concentrations of 0%, 20%, or 40%) at 37°C for 60 minutes. After incubation, the reaction mixture was diluted to 1/5.4 with Hepes-Tyrode's buffer (KCl, 2 mM; NaCl, 127 mM; NaH_2PO_4, 0.5 mM; glucose, 5.6 mM; $NaHCO_3$, 12 mM; HEPES, 5 mM; 0.35% BSA; pH 7.3). Eighteen microliters of the diluted reaction mixture was added to 18 μl of a cocktail of FITC-conjugated PAC-1, PE-conjugated anti-CD62P and PerCP-conjugated anti-CD42a. FITC-conjugated anti-mouse IgM, PE-conjugated anti-mouse IgG, and PerCP-conjugated anti-mouse IgG were used as negative controls. All antibodies were purchased from BD bioscience-Pharmingen, San Jose, CA. The reaction mixture was then incubated with 4 μl of ADP (final concentration of 0, 0.05, 0.1, 0.5, 5, or 10 μM) for 20 minutes at room temperature in the dark. After incubation, the platelet suspension was fixed with 500 μl of paraformaldehyde (final concentration, 1%) and washed once with PBS. Finally, the platelets were resuspended in

500 μL of PBS. The samples were analyzed by flow cytometry (LSR, Becton-Dickinson, San Jose, CA). Fluorescence data from 10,000 platelet events were collected in logarithmic mode. The platelet population was identified by the number of CD42a-positive events.

Assay of Mediator Release

The platelet mediator release assay was carried out as described by Santos et al. [17], with slight modifications. Platelet-rich plasma (PRP) was obtained from citrated venous blood of unselected healthy subjects by centrifugation (140 g, 15 minutes, 22°C), and 600 μl of PRP (final concentration, 1.7×10^8/ml) was incubated with 400 μl of HbV (0%, 20%, or 40%) at 37°C for 60 minutes. After incubation, the mixture was divided into two 480 μl aliquots. For the collagen-induced platelet release reaction, the mixture was activated with 20 μl of collagen (final concentration, 1 μg/ml) (NYCOMED ARZNEIMITTEL BMBH, Germany) or buffer at 37°C for 5 minutes. For the ADP-induced platelet release reaction, the mixture was activated with 20 μl of ADP (final concentration, 2 μM) (SIGMA) or PBS at room temperature for 20 minutes. After incubation, the tube was centrifuged at 10,000 g for 1 minute. The cell-free supernatant was then transferred to another tube and centrifuged at 10,000 g for 30 minutes. The cell-free supernatant was stored at −20°C until the measurement of platelet release. Commercially available enzyme-linked immunosorbent assays (ELISAs) were used to measure the levels of RANTES (R&D Systems, Minneapolis, MN), serotonin (ICN Biomedicals Inc., Costa Mesa, CA) and TXB_2 (Cayman Chemical Company, Ann Arbor, MI) in duplicate experiments, according to the manufacturers' recommendations. Enzyme immunoassays were used to measure the levels of β-TG (Asserachrom β-TG, Roche Diagnostics, Tokyo, Japan).

Statistical Analysis

A two-way repeated measures ANOVA with Bonferroni correction was used for multiple comparisons of mediator levels and surface marker levels among different concentrations of HbV. A p value <0.05 was considered to indicate a significant difference.

RESULTS

Effect of HbV on the Binding of PAC-1 and the Expression of CD62P on Resting and ADP-stimulated Platelets In Vitro in Whole Blood

First, the effect of HbV on the binding of PAC-1 to platelets and the surface expression of CD62P on platelets with or without ADP stimulation

was examined in a whole blood environment in vitro. Without ADP stimulation, PAC-1 binding to platelets was discernible. Preincubation of whole blood with 20% or 40% HbV alone did not cause a significant difference in PAC-1 binding to the platelets. Stimulation of platelets with varying concentrations of ADP caused a gradual increase in the percentage of PAC-1 positive cells (Fig. 1A). Preincubation of whole blood with 20% or 40% HbV resulted in a slight, but significant, enhancement in the percentage of PAC-1 positive cells, compared to the results of comparable experiments without HbV, at ADP concentrations ranging from 0.05 μM to 5 μM (Fig. 1A).

Unstimulated platelets showed only a slight expression of CD62P, regardless of HbV treatment (Fig. 1B). The treatment of platelets with varying concentrations of ADP also led to gradual increases in the percentage of CD62P-positive cells, but preincubation of whole blood with 20% or 40% HbV did not affect the ADP-induced increase in the percentage of CD62P-positive cells (Fig. 1B).

Effect of HbV on Secretion of Platelet-derived Mediators in Resting and ADP-stimulated Platelets In Vitro

Next, the effect of HbV on the release of mediators from platelets stimulated with or without a submaximal dose of ADP, a weak platelet

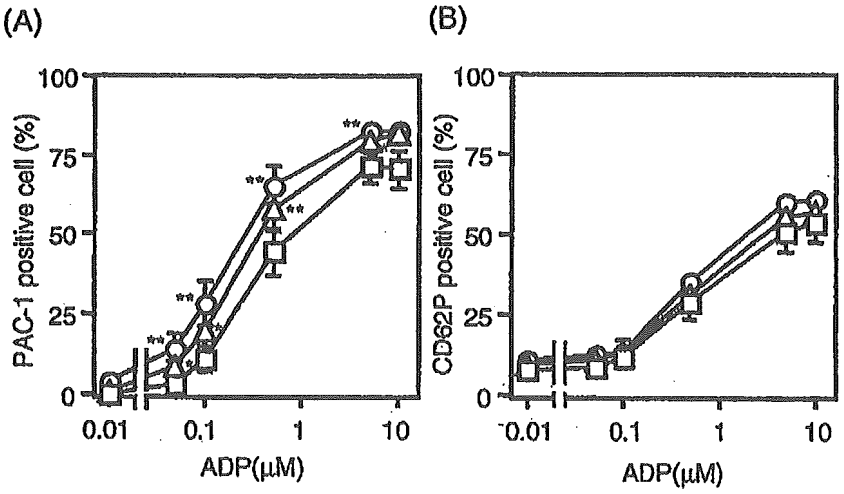

Figure 1. Effect of HbV on platelet surface activation markers. (A) PAC-1 binding to platelets and (B) CD62P expression on platelets. Whole blood was incubated with HbV at concentrations of 0% (square), 20% (triangle), or 40% (circle). Whole blood was then stimulated with or without various concentrations of ADP, as described in the Materials and Methods section. Values are the means ± SE of 4 experiments. *$p < 0.05$, **$p < 0.01$, compared with control (0% HbV).

agonist, was examined. Without ADP stimulation, a slight, but significant, reduction in the spontaneous release of TXB_2 from platelets pretreated with 40% HbV was observed (Fig. 2A). Similarly, the levels of spontaneous release of RANTES from platelets pretreated with both 20% and 40% HbV were slightly, but significantly, reduced in comparison with those from platelets that were not pretreated with HbV (0% HbV). The treatment of PRP with ADP caused a significant increase in the levels of each mediator in the releasates. Pretreatment of PRP with either 20% or 40% HbV did not affect the ADP-induced release of each mediator, although a slight reduction was observed in each case (Fig. 2).

Effect of HbV on Secretion of Platelet-derived Mediators in Resting and Collagen-stimulated Platelets In Vitro

The effect of HbV on mediator release was further examined using platelets stimulated with or without collagen, a strong platelet agonist. Without collagen stimulation, the levels of serotonin, TXB_2, and β-TG were not affected in the cell-free releasates from PRP after pretreatment with either 20% or 40% HbV, although the RANTES levels were slightly, but significantly, reduced ($p < 0.05$) (Fig. 3). Collagen stimulation of the PRP caused a marked increase in the levels of each mediator but pretreatment with 20% or 40% HbV did not affect the collagen-induced release of TXB_2, RANTES, or β-TG. The levels of serotonin in the collagen-stimulated PRP tended to decrease in an HbV-dose dependent manner.

DISCUSSION

In this study, the effect of HbV on the expression of platelet activation markers in the presence or absence of platelet agonists was evaluated in vitro. Integrin $\alpha_{IIb}\beta_3$ mediates platelet adhesion and aggregation and plays a crucial role in thrombosis and hemostasis [18]. $\alpha_{IIb}\beta_3$ is expressed in a low affinity state on resting platelets. On platelet activation, $\alpha_{IIb}\beta_3$ shifts to a high affinity conformation that efficiently binds its ligands, including fibrinogen and von Willebrand factor. Thus, such activation is a prerequisite for fibrinogen binding to platelets, which culminates in platelet aggregation. The high affinity conformation of $\alpha_{IIb}\beta_3$ on human platelets can be detected by the monoclonal antibody PAC-1 [15, 16, 19]. Because low doses of ADP cause an increase in PAC-1 binding within a short time period, this phenomenon is regarded as one of the earliest events in platelet activation, and PAC-1 has been shown to be a highly sensitive and specific marker of platelet activation [15, 16].

Activated platelets secrete a number of prothrombotic substances, like TXA_2, serotonin, and CD62P that act synergistically to form thrombi. TXA_2 is synthesized via the cyclooxygenase-mediated arachidonic

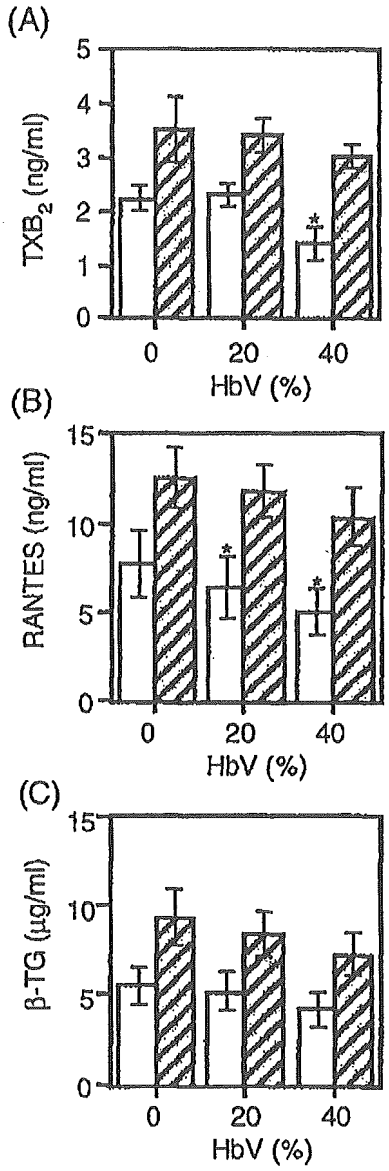

Figure 2. Effect of HbV on ADP-induced platelet mediator release. ADP-induced release of (A) TXB_2, (B) RANTES, and (C) β-TG from human platelets. PRP was incubated with concentrations of 0%, 20%, or 40% HbV and then stimulated with (hatched columns) or without (open columns) ADP, as described in the Materials and Methods section. Values are the means ± SE of 5 (A) and 6 (B, C) experiments using blood from different donors. *$p < 0.05$, compared with control (0% HbV).

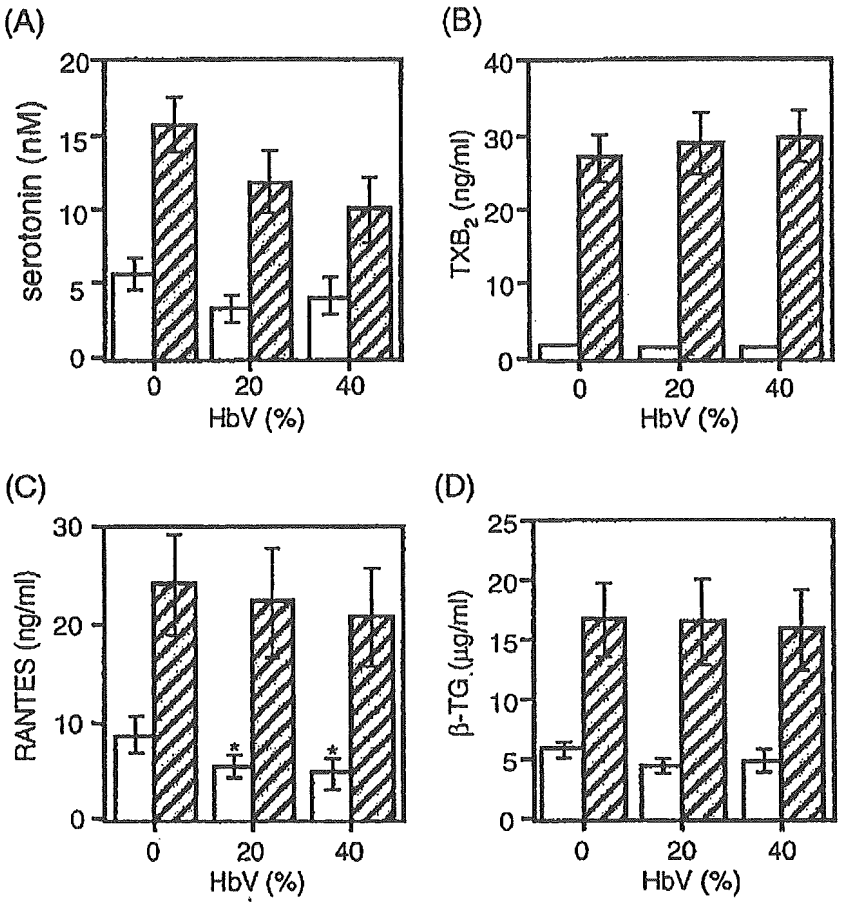

Figure 3. Effect of HbV on collagen-induced platelet mediator release. Collagen-induced release of (A) serotonin, (B) TXB_2, (C) RANTES, and (D) β-TG from platelets. PRP was incubated with concentrations of 0%, 20%, or 40% HbV and then stimulated with (hatched columns) or without (open columns) collagen, as described in the Materials and Methods section. Values are the means ± SE of 5 experiments using blood from different donors. *$p < 0.05$, compared with control (0% HbV).

metabolic pathway [20] and is a potent platelet agonist that induces a rapid positive feedback loop, thereby amplifying the activation signals and enabling robust platelet recruitment at the site of vascular injury [21]. Serotonin is a bioactive amine that localizes in dense granules of resting platelets and is secreted upon platelet activation. Serotonin also has a prothrombotic effect on platelets [12]. Interactions between platelets via CD62P stabilize the initial $\alpha_{IIb}\beta_3$–fibrinogen interactions, thereby promoting the formation of large, stable platelet aggregates

[22]. In addition, CD62P is the major surface receptor for neutrophils and monocytes on activated platelets, mediating leukocyte adhesion. Thus, platelet CD62P is involved in the recruitment of both platelets and leukocytes into an evolving thrombus [22–25].

Recent studies have extended platelet function to include the modulation of local inflammatory events through the release of chemokines and cytokines [13]. RANTES and β-TG [13] are stored in α-granules in platelets and are released from platelets on activation. RANTES has diverse inflammatory effects, such as histamine release from basophils and the exocytosis of eosinophil cationic protein. Furthermore, RANTES is a powerful chemoattractant for T cells, basophils, and eosinophils [13, 26]. β-TG, a platelet-derived CXC chemokine, is also released into the blood at micromolar concentrations and plays an important role in the recruitment of neutrophils to sites of tissue injury [27]. Consequently, the aberrant release of serotonin, TXA_2, RANTES, or β-TG in response to the inappropriate activation of platelets could result in pathological thrombosis, inflammatory reactions, or allergic responses.

The present study demonstrated that the exposure of blood samples to HbV at concentrations of up to 40% did not cause platelet activation, as measured by various markers, although the levels of RANTES and TXB_2 were significantly, but marginally, reduced. In terms of the effect of HbV on platelet activation triggered by submaximal concentrations of agonists, the enhancement of PAC-1 binding to platelets, one of the earliest markers of activation, was observed after the exposure of blood samples to HbV at concentrations of up to 40%, suggesting that HbV might have an enhancing effect on agonist-induced platelet aggregation. Other than the slight enhancing effect of HbV on PAC-1 binding in the presence of agonist stimulation, however, none of the other parameters were significantly affected. Rather, the levels of TXB_2, RANTES, and β-TG tended to be reduced by ADP stimulation, while the level of serotonin tended to be reduced by collagen stimulation. Thus, the lack of coordinated potentiation in the levels of prothrombotic substances (i.e., TXB_2 and serotonin) and stabilizing molecules involved in the initial $\alpha_{IIb}\beta_3$–fibrinogen interactions (i.e., CD62P) in response to the presence of an agonist suggests that the enhancing effect of HbV on platelet reactivity to agonists, if present, is not likely to lead to the deleterious formation of thrombi. In addition, the absence of adverse effects on the secretion of RANTES and β-TG suggest that HbV is unlikely to trigger the initiation and/or aberrant perpetuation of inflammatory and allergic reactions.

The present results are of value for estimating the biocompatibility of HbV and human platelets. Further research is warranted to investigate whether the administration of HbV has any effect on platelet activation and platelet functions in vivo.