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総　説

ヘモグロビン小胞体のin vitroにおけるヒト血液細胞および血漿タンパクへの適合性

Biocompatibility of Hemoglobin Vesicles, a Cellular-type Artificial Oxygen Carrier, on Human Blood Cells and Plasma Proteins in Vitro

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和文抄録

ヘモグロビン小胞体（hemoglobin vesicles; HbV）は，脂質二重膜からなるリポソームの中にヘモグロビンを内包させたセル型の人工酸素運搬体である．HbVはいわゆる「人工赤血球」として血管内に輸注されるため，その臨床応用に向けた安全性の評価には，生体適合性，とくに血液構成成分に対する適合性をみることが重要である．そこで我々はヒト血液細胞およびヒト血漿タンパク系を用いたin vitroの評価に焦点をあてた検討をおこなってきた．止血や炎症反応に関与する血小板に対し，HbV自体には活性化作用はみられず，またアゴニストによる活性化への影響もみられなかった．自然免疫に重要な働きをする好中球の機能（走化能，脱顆粒，活性酸素産生）の活性化に対しても，HbVは影響を与えなかった．造血機能に対しては，HbVはin vitroでの共存が3日間までの短期間であれば造血前駆細胞活性を損なうことはないことが示された．またリポソームの特性から補体の活性化が懸念されたが，現行のHbVでは活性化はみられず，さらに凝固系，カリクレイン・キニン系にも影響はみられなかった．以上のことから，HbVはヒト血液細胞ならびに血漿タンパクに対し高い適合性を持つことが示唆される．

Abstract

Hemoglobin vesicles (HbV), a cellular-type artificial oxygen carrier, are composed of human hemoglobin encapsulated within a phospholipid bilayer membrane. As HbV are injected intravenously, biocompatibility of the HbV with blood components is very important to ensure safety of this material for clinical use. We have evaluated this biocompatibility by focusing on the influence of HbV on human blood cells as well as plasma proteins in vitro. 1) As to the influences to platelets which are involved not only in the hemostasis but also in inflammation, HbV themselves did not activate platelets, and had no aberrant effect on agonist induced platelet activation. 2) HbV did not affect on agonist-induced activation of neutrophil functions (chemotaxis, degranulation, and production of superoxide) which play important roles on innate immunity. 3) HbV had no effect on the hematopoietic progenitor activity, if the exposure period is brief. 4) The present HbV (containing DHSG) did not activate complement system, although the old-type HbV (containing DPPG, no PEG modification) did markedly. 5) The coagulation as well as kallikrein-kinin cascades were not affected by the present HbV. Thus, our in vitro studies show that HbV are highly biocompatible with human blood cells and human plasma proteins.

Keywords

artificial oxygen carrier, biocompatibility, platelets, neutrophils, hematopoietic progenitor cells, complement, coagulation, kallikrein-kinin

はじめに

赤血球製剤の人工代替物として人工酸素運搬体の開発が進められてきている．人工酸素運搬体の臨床応用における利点は，輸血時の血液型判定の必要がないこと，製剤の長期保存が可能となること，同種抗原感作や細菌感染およびウイルス感染の伝播を回避できること等があげられる[1]．これらの利点から，災

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害時の緊急事態において大量の血液製剤が必要とされる時，また血液型検査および適合した血液製剤の供給を待機する間，人工酸素運搬体を応急措置として用いることが想定される．現行の同種血輸血や自己血輸血との組み合わせにより，人工酸素運搬体は合理的な輸血システムを構築することに寄与することが期待される．

ヘモグロビン小胞体（hemoglobin vesicles; HbV）は，粒子径約250nmのリポソーム包埋型ヘモグロビンで[1]，細胞膜を模擬し人工的に作成したリン脂質／コレステロール／陰性荷電脂質／PEG脂質からなる脂質二重膜のリポソームの中にヘモグロビンを内包させたセル型の人工酸素運搬体である．HbVの人工赤血球としての酸素運搬能に関する有効性，組織分布，代謝等についてはこれまで詳細に検討されている[1]．一方，HbVの臨床応用を進めていく上では，その生体適合性を評価することが重要である．生体適合性を評価すべき項目は多岐にわたる[2]．その中で我々は，HbVが輸注された際に相互作用を起しうる血液構成成分，即ち，血液細胞および血漿タンパクに対しHbVがどのような影響をあたえるかについて焦点をあてた検討をおこなってきた．本稿においてはヒト血液細胞として血小板，好中球，造血前駆細胞を，ヒト血漿タンパクとして補体系，血液凝固系，カリクレイン-キニン系をとりあげ，HbVのin vitroにおけるそれぞれへの影響について我々の検討結果を概説し，HbVの生体適合性について言及したい．

1．ヘモグロビン小胞体の血小板活性化に対する影響

げっ歯類の動物モデルにおいては，リポソーム包埋型ヘモグロビン投与による副作用が知られ，その症状の一つとして一過性の急性血小板減少が報告されている[3-5]．同様な一過性の急性血小板減少は，陰性荷電のリポソームの投与においてもみられている[6,7]．この急性血小板減少の発生原因を解明するため，in vitroにおいてリポソームとげっ歯類の血小板との結合に関して研究がなされた結果[8]，リポソームの構成成分が負電荷の脂質である場合，ラット多血小板血漿とのインキュベーションによりリポソームは血漿中の補体成分C3bに覆われ，C3bを介したリポソーム - C3b - 血小板上CR1 receptorの結合による凝集塊が形成されることが示された．そしてin vivoにおいては，この凝集塊が速やかに網内系によって処理されることが急性血小板減少の原因と考えられている[8]．これに対しヒト血小板上にはCR1 receptorが存在しないため，リポソームと血小板との凝集塊は観察されない[8]．従ってヒトにおいてはリポソームの構成成分が負電荷の脂質であったとしても，動物モデルで観察されるリポソーム輸注後の急性血小板減少は発症しないと推測される．

一方，血小板はその顆粒中に種々の炎症性生理活性物質を含有していることや，膜表面の接着分子を介して白血球と結合することから，止血血栓の形成のみならず炎症反応にも関与していると考えられている[9,10]．HbVの投与により急性の血小板減少症が起こらないとしても，HbVが生体内で血小板を活性化したり，あるいはアゴニストによる反応を助長させたりするような影響を及ぼし，血小板由来生理活性物質の放出が起こるという事態は，生体にとって好ましいことではない．

このような背景のもと，われわれはリポソームの構成成分としてDPPG（1,2-dipalmitoyl-*sn*-glycero-3-phosphatidyl glycerol）を含有するHbVの血小板活性化への影響について，RANTESの放出反応を指標として検討した[11]．RANTESは血小板のα顆粒に存在する炎症性ケモカインで，好塩基球，好酸球，単球およびリンパ球に対する強力な走化作用を有する[9,12]．そこでヒト多血小板血漿と最大20％（vol/vol）までの濃度のHbVを予めインキュベーション後，アゴニストであるコラーゲンで刺激した反応上清中のRANTESの濃度の増加を測定した．その結果，コラーゲンの濃度に依存したRANTESの放出がみとめられたが，20％ HbVと予めインキュベーションさせた場合においては，RANTESの放出への影響はみられなかった（Table 1）．またアゴニスト刺激なしの場合においても，RANTESの放出に関して血小板とHbVを予めインキュベーションした影響はなんらみとめられないことから，HbVが血小板を活性化する作用もないと考えられた．

次に，リポソームの構成成分としてDPPGをカルボン酸脂質DHSG（1,5-*O*-dihexadecyl-*N*-succinyl-L-glutamate）に置き換えた現行のHbVの血小板への影響について，HbV濃度最大40％まで検討した[13]．血小板活性化の評価は，RANTESに加え同じくα顆粒に存在する炎症性ケモカインβ-thromboglobulin（β-TG），濃染顆粒に存在するセロトニンの放出反応，ポジティブフィードバックによって血小板活性化を増幅するThromboxane A_2（TXA_2）の産生，さらにα顆粒由来のCD62Pの膜表面への発現，および血小板膜糖タンパクαIIbβ3の構造変化を認識する抗体PAC-1の結合を指標とした．なお，TXA_2は半減期が短いためその代謝産物のThromboxane B_2（TXB_2）を測定した（Table 1）．

Table 1. Effect of HbV on human platelets.

Index	Stimulant	Type of HbV	Conc. of HbV	Effect
RANTES release	Collagen (+)	DPPG-HbV	≦ 20%	No effect
	(-)	DPPG-HbV	≦ 20%	No effect
RANTES release	Collagen (+)	DHSG-HbV	≦ 40%	No effect
	(-)	DHSG-HbV	≦ 40%	Marginal reduction
β-TG release	Collagen (+)	DHSG-HbV	≦ 40%	No effect
	(-)	DHSG-HbV	≦ 40%	No effect
Serotonin release	Collagen (+)	DHSG-HbV	≦ 40%	No effect
	(-)	DHSG-HbV	≦ 40%	No effect
TXB_2 production	Collagen (+)	DHSG-HbV	≦ 40%	No effect
	(-)	DHSG-HbV	≦ 40%	No effect
CD62 expression	ADP (+)	DHSG-HbV	≦ 40%	No effect
	(-)	DHSG-HbV	≦ 40%	No effect
PAC-1 binding	ADP (+)	DHSG-HbV	≦ 40%	Slight potentiation
	(-)	DHSG-HbV	≦ 40%	No effect

セロトニンは血小板の濃染顆粒に存在する生理活性アミンで，血管収縮作用をはじめ繊維芽細胞増殖作用，マクロファージの活性酸素産生促進作用，NK細胞活性化促進作用を有する[9]．CD62Pはセレクチンファミリーに属する接着分子で[9]，血小板

活性化に伴ってα顆粒から膜表面に分布するため，血小板活性化マーカーとして汎用されている[11]．PAC-1は血小板上のαIIbβ3（GPIIb-IIIa）が血小板活性化にともなって構造変化を起こした時に発現するエピトープを認識するモノクローナル抗体である[12,13]．低濃度のADP刺激によっても発現の増加がみられることから，高感度でかつ特異的な血小板活性化マーカーとして有用であるとされている．αIIbβ3はインテグリンファミリーに属する接着分子であり，血小板においてフィブリノーゲンやvon Willbrand因子のレセプターとして機能し，血小板凝集や粘着の重要な分子である[16]．

ヒト多血小板血漿に20％および40％の濃度のHbVを1時間予めインキュベーション後，アゴニスト刺激の無い場合は，血小板反応上清中のRANTES，セロトニン，β-TG，TXB_2および血小板表面CD62Pのレベルの上昇はみられないことから，HbVに血小板活性化作用は無いと考えられた．この際，RANTESレベルはむしろ減少したが，この程度の変化での臨床的意義は少ないと推察された．アゴニスト刺激により血小板の脱顆粒を惹起させると，いずれの濃度のHbV処理においてもRANTES，セロトニン，β-TGの放出，TXB_2の産生，およびCD62P発現が引き起こされ，そのレベルはHbV未処理のものと有意差はみられなかった（Table 1）．一方，PAC-1の発現はHbV未処理では影響がないものの，HbV処理により低濃度のADP刺激血小板において僅かな亢進がみられた．PAC-1の発現の亢進は空の小胞体処理においても観察されたため，ヘモグロビンの有無にはよらず，小胞体それ自体または小胞体の構成成分に起因する可能性が考えられた．以上のことから，HbVは低濃度のアゴニスト存在下でαIIbβ3の構造変化を僅かに促進するものの，CD62Pの膜への発現，RANTES，セロトニン，β-TGの放出，TXB_2の産生にはアゴニスト刺激の有無にかかわらず影響を与えないことから，ヒト血小板に対し高い生体適合性があると結論された（Table 1）．

2．ヘモグロビン小胞体の好中球活性化に対する影響

HbVがヒトに投与される状況として大量出血が想定される．その際に，生体防御のファーストラインを担う好中球の機能に対し，HbVが抑制的に働くならば，感染症などを合併する危険性が懸念される．循環血中の好中球は活性化を受け，炎症部位に遊走し，生体を脅かす細菌を効率的に殺菌する必要がある．好中球は，ザイモザンや細菌由来のペプチド（*N*-formyl-methionyl-leucyl-phenylalanine（fMLP））によって活性化を受け，多岐にわたる機能を発揮することが知られているが，PEG-distearoylphosphatydylethanolamine（PEG-DSPE）修飾を受けたある種のリポソームは，これらの刺激に対する好中球の反応を阻害することが報告されている[7]．逆に，フォスファチジルコリンやフォスファチジルセリンからなるリポソームが，マウスのアレルギーモデルにおいて，好中球の肺への集積を昂進させたという報告がなされている[8]．そこで，HbV自体がPEG-DSPE修飾をうけていることもあり，fMLP刺激によって好中球が発揮する機能に対し，どのような影響を及ぼすかについて検討を加えた[9]．

Table 2 Effect of DPPG-HbV on fMLP-induced neutrophil functions

Function	Conc. of HbV	Effect
Chemotaxis	≤ 0.6%	No effect
Upregulation of CD11b expression	≤ 0.6%	No effect
Degranulation of Gelatinase B	≤ 6%	No effect
Superoxide production	≤ 6%	No effect

この検討においては，リポソームの構成成分としてDPPGを含有するHbVを用いた．fMLP刺激による好中球の機能評価として，(1) 好中球の走化能，(2) β_2インテグリンであるCD11bの膜発現，(3) 脱顆粒にともなうgelatinase B（matrix metalloproteinase-9）の放出，(4) 活性酸素の産生をとりあげた．Table 2にこれらの結果を示した．

走化性（ケモタキシス）は，細胞が化学物質の濃度勾配に従い，一定の方向に運動性を示すことであり，ヒト単離好中球を異なる濃度（最大0.6％）のHbVと37℃にて30分間予めインキュベーション（プレインキュベーション）後，ケモタキシス・チャンバー法によって評価をおこなった．fMLP（1 μM）刺激によって好中球の走化能が活性化されたが，これに対するHbVの影響はみとめられなかった．CD11bはCD18と二量体を形成して好中球の遊走能における接着因子Mac-1として機能する分子であるが，HbVとプレインキュベーションさせた好中球において，fMLP刺激によるCD11bの発現亢進は，HbVを作用させなかった好中球の場合と同等であった．gelatinase Bは，好中球の組織浸潤に関与する酵素であるが，fMLP刺激による好中球からの放出の経時変化をHbVとのプレインキュベーションの有無で比較したが，両者には違いはみとめられなかった．また好中球の殺菌能に関与する活性酸素産生についても，HbVとプレインキュベーションした好中球からのfMLPによる産生量は，HbVを作用させなかった好中球の場合と同等であった．以上の結果より，fMLP刺激によって発揮される好中球の少なくとも4つの機能において，用いたHbVの濃度ではなんら影響はみられず，HbVの好中球への高い適合性が示唆された．

PEG-DSPE修飾したリポソームがfMLP刺激による好中球の走化能活性化を阻害するという報告[7]を上述したが，リン脂質濃度にして大過剰の濃度を用いたにもかかわらず，HbVでは阻害がみられないことの理由は不明である．リポソームの構成成分であるリン脂質の種類，脂肪酸の飽和度の程度，脂肪酸の側鎖の数が，fMLPやザイモザンによる好中球の走化能に影響することが報告されている[20]．前述のPEG-DSPE修飾したリポソームとHbVとの間では，これらのパラメーターを始め，PEG-DSPEとリポソームのモル比が異なっており，これらの違いが異なる結果を得た理由となっている可能性が考えられる．

以上，HbVの好中球への高い適合性が示されたが，より高濃度即ち臨床で用いられる20％および40％での検討をみる必要性は残されている．

3．ヘモグロビン小胞体の造血前駆細胞に対する影響

血管内投与したリポソーム製剤は，漸次，肝臓のクッパー細胞，脾臓および骨髄のマクロファージなどの細網内皮系において捕捉される[21]．HbVにおいても，アイソトープラベルを用いた追跡によって組織分布が詳細に検討され，肝臓，脾臓，骨髄に主として分布していることが示されている[22]．他の組織学的検討においても，HbVは細網内皮系において捕捉されることが報告されている[23]．骨髄にHbVが移行することから，骨髄を主たる場とする造血に対し少なからず影響するのではないかと考えられた．40％脱血しHbVを輸血したラットさらにビーグル犬の場合，脱血による赤血球造血の亢進によってヘマトクリットは1週間以内には回復し，特に造血系への影響は認められていない[24,25]．さらに，20％のHbVをtoploadさせたラットの場合においても，末梢血の血球数は投与後1週間観察しても一定に保たれていた[26]．とはいえヒトへの投与を考えた場合，その造血前駆細胞の増殖・分化への影響について，in vitroの系において検討する必要があると考えられ検討をおこなった[27]．

造血のin vitroのアッセイ系としては，コロニー形成細胞の数を評価するコロニーアッセイと造血前駆細胞の増殖の度合いを評価する液体培養系を用いた．そして臍帯血から分離したCD34陽性細胞を含む単核球を，造血を支持する各種サイトカインを添加したメチルセルロースからなる半固形培地に播種し，一定期間培養後に形成したコロニーの数をカウントした．赤芽球バーストとよばれるBFU-E（Burst forming unit of erythrocyte），顆粒球・マクロファージ系コロニーとよばれるCFU-GM（Colony forming unit of granulocyte and macrophage），両者が混在したCFU-mix．これらのコロニーの総数であるColony-forming cells（CFC）は培養2週間後に，増殖能が高くコロニーのサイズが1 mm以上になったものをHPP-CFC（High proliferative potential-colony forming cell）と定義し，培養4週間後にそれぞれカウントした．用いたHbVはPEG修飾されたDHSGを構成成分とするHbVで，その濃度は最大3％と設定した[21]．

コロニーアッセイの培地にHbVを添加し，14日間共存させると，それぞれのコロニーの数およびサイズがHbVの濃度に依存して減少する傾向がみられた．さらに14日間HbVと共存後にコロニー数を測定するHPP-CFCの数は著しく減少した（Fig. 1A）．一方，HbVにCD34陽性細胞を含む単核球を20時間だけ曝してコロニーアッセイした場合は，HPP-CFCの数に影響はなく，3日間曝した場合でも，HPP-CFCの数の減少の程度は少なかった（Fig. 1B）．次に液体培地に血清と造血支持サイトカインを添加して，赤芽球系または顆粒球系の細胞へと増殖させる系にHbVを添加させ影響を評価した．10日間の培養期間，HbVが継続して存在する場合には赤芽球系または顆粒球系への細胞の増殖が抑制された．これに対し，コロニーアッセイの場合と同様に，HbVに20時間または3日間曝しただけの場合は，赤芽球系または顆粒球系への細胞の増殖は影響されなかった（Table 3）．

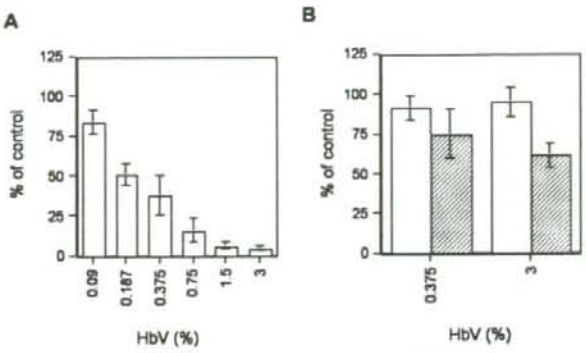

Fig. 1. Effects of HbVs on the formation of high proliferative potential colony forming cells (HPP-CFC) in the clonogenic assay of cord blood-derived hematopoietic cells. Cord blood-derived mononuclear cells were seeded at 300 at CD34 cells per dish in complete methylcellulose medium for human clonal culture assays. HPP-CFC was scored after 28 days incubation. (A) HbVs (0 to 3％) were added to the cells until the end of culture. (B) Cord blood-derived mononuclear cells were incubated for 20 h (open column) or for 3 days (slashed column) with different concentrations of HbVs in IMDM containing FCS, BSA, and cytokines. Subsequently, they were recovered, washed to remove the HbVs, and subjected to the clonal assay. Data are expressed as the mean ± SD of the percentage of control (HbV 0％) performed on three separate cord blood donors in (A) and (B).

以上HbVは，in vitroの閉鎖系において，長期間にわたり共存することにより造血前駆細胞の活性を阻害するという結果が，2つの評価系から明らかとなった．しかしながら，動物モデルでHbVを投与した場合，骨髄において1-3日にHbVの蓄積が認められ，以後1週間では激減することが報告されている[20]．

Table 3. Effect of DHSG-HbV on the proliferation of erythroid and myeloid lineage cells in liquid culture.

Exposure period to HbV	CD235a⁺ cells			CD15⁺ cells		
	HbV conc.(%)			HbV conc. (%)		
	0.75	1.5	3.0	0.75	1.5	3.0
20h	93.7 ± 10.0	94.9 ± 1.2	92.2 ± 8.8	100.8 ±14.3	96.3 ±7.9	96.6 ±13.3
3 days	85.2 ± 22.3	92.6 ± 11.5	89.0 ± 14.5	92.9 ± 6.1	95.8 ± 5.4	91.7 ± 4.7
10 days	47.5 ± 16.6**	46.6 ± 18.2**	27.3 ± 23.4**	65.2 ± 20.3*	55.2 ± 18.6*	37.2 ± 22.8*

Various concentrations of HbVs were added to the medium containing the cord blood-derived CD34⁺ cells. After 10 days' incubation, CD235a⁺ cells for erythroid lineage and CD15⁺ cells for myeloid lineage, respectively, were analyzed by flow cytometry. The number of CD235a⁺ cells or CD15⁺ cells at each concentration of DHSG-HbV is expressed as a percentage of the number in the control (HbV 0%). Data are represented as the mean ± SD from three experiments performed on three separate cord blood donors. *$p < 0.05$, **$p < 0.01$ versus HbV (0%).

よって，2週間連続してHbVに造血幹・前駆細胞が曝されるという状況はヒトの臨床例では想定できないし，むしろ1-3日間曝されるというのが想定できる期間である．さらにin vivoでは，HbVはマクロファージ内に蓄積しており，in vitroのようにHbVと造血幹・前駆細胞が直接相互作用をすることは極めて少ないと考えられる．従って造血幹・前駆細胞が直接1-3日間HbVに暴露される場合において，造血前駆細胞の活性に大きな影響がなかったという結果は，HbVをヒトに投与した場合，造血に大きな悪影響を及ぼす可能性が極めて少ないことを支持するものと捉えることができる．

国外では，ヘモグロビンを基盤とし化学修飾させたセルフリー型の人工酸素運搬体の開発が進められている．これらの修飾ヘモグロビンや分子内架橋を行ったヘモグロビンは，我々と同じようにヒト臍帯血から調製したCD34陽性細胞を用いた液体培養系において，赤芽球前駆細胞の増殖の亢進を促すと報告されている[28]．また，AIDSの治療に用いられる薬剤3'-azido-3'-deoxythymidine（AZT）による造血抑制を，ヘモグロビンおよび化学修飾ヘモグロビンがin vitro，in vivoの両方で回復させる（但し赤血球造血に特異的）ことが示され[29,30]，これらの赤血球造血作用は酸素運搬能以外の付加価値と位置付けられている．修飾ヘモグロビンのこれらのin vitroでの作用は，赤芽球系前駆細胞が修飾ヘモグロビンを取り込み，赤血球造血作用のあるhemeに分解するためと推測されている[29]．HbVの場合は，赤芽球系前駆細胞を含めて造血前駆細胞の増殖亢進作用はみられず，逆に長期間では抑制する結果となったが，その機序は現時点では明らかでない．少なくとも赤芽球系前駆細胞は，HbVを修飾ヘモグロビンのようには容易には取り込まないと考えられる．

4．ヘモグロビン小胞体の補体系，凝固系，カリクレイン-キニン系に対する影響

リポソームは細胞膜と同様リン脂質を構成成分としているが，生体にとっては異物であり，血液細胞のみならず血漿タンパク質と相互作用する[31]．この作用は，リポソームの大きさ，荷電，構成脂質，さらにリポソーム自体への化学修飾により異なってくる．リポソームの中で陰性荷電のものは，ラット，モルモット，そしてヒトの補体を活性化することが広く知られている[32-34]．その結果，オプソニン化したリポソームは，細網内皮系により循環血液中から速やかに排除される．また陰性荷電リポソームは凝固系第7因子を活性化させ，内因系の凝固カスケードならびにカリクレイン・キニン系活性のトリガーとなる[35,36]．リポソームの構成成分のコレステロール含量も補体の活性化に影響することが知られ，この活性化は血液中の自然抗体によると考えられている[31,37,38]．これまで述べてきたリポソーム包埋型ヘモグロビンにおいても，ヒトの血液中の脂質に対する自然抗体によって，古典経路と第二経路の両方を介して補体の活性化が惹起される[39]．さらに，ブタモデル実験においては，ある種のリポソームによって偽アレルギー反応が起こり，補体活性化との関連が考えられている[40-42]．一方，リポソームをPEG修飾することで，これらの反応を防ぐことができることが報告されている[43-45]．しかしながら，リポソーム包埋型ヘモグロビンのみならず抗ガン剤doxorubicinを封入したPEG修飾リポソームDoxil/Caelyxにおいても，顔面紅潮，呼吸困難，紅斑，胸痛，背面痛，血圧低下または血圧上昇が報告されている[46-48]．またこれらの症状はdoxorubicin自体では起こらないため，リポソームによるものと考えられている[48]．したがってHbVの生体適合性を評価するうえで，ヒトの補体系，凝固系，カリクレイン-キニン系に対する影響を検討することの意義は大きいと考えられた．

この検討においては，DHSGをそのリポソーム構成成分としPEG修飾された現行のHbV（DHSG-HbV），DPPGを構成成分としPEG修飾されたHbV（DPPG-HbV），DPPGを構成成分とするがPEG未修飾のHbV（DPPG-HbV（no PEG）），強陰性荷電PEG未修飾リポソーム（EL-A）を用いた[20]．なお，EL-Aの脂質構成はDPPC:CHOL:DPPG（30:40:30）（mol％）からなり，その生理食塩水中でのゼータ電位は-47.2mVであった[20]．

HbVを健常人ヒト血清と20％あるいは40％で混合し，37℃にて24時間インキュベーション後，遠心により各種HbVを除去し，その上清の補体価（CH50）を測定した．Table 4に示すように，DHSG-HbVを混合することによって血清が希釈されるためCH50は低下した．強陰性荷電PEG未修飾リポソーム（EL-A）を添加した群では著しいCH50の低下がみられるのに対し，DHSG-HbVを20％あるいは40％で添加した群では，それぞれ生食を添加した場合のCH50より減少することはなかった．さらに3種のHbVで比較してみると（Table 5），DHSG-HbVとDPPG-HbVは，補体消費を起こさなかったのに対し，DPPG-HbV（no PEG）は40％の添加によってほぼ完全に補体が消費された．以上の結果から，現行のDHSG-HbVおよびPEG修飾されたDPPG-HbVは，ヒト血清補体系を活性化しないと考えられた．

Table 4. Consumption of complement by HbV and Liposome.

Additive	CH50 (U/mL) (additive:serum) 20:80	40:60
Saline	33.4 ± 2.8	21.4 ± 1.7
DHSG-HbV	33.5 ± 2.9	22.9 ± 2.4
EL-A	25.1±2.7*	5.9 ± 0.7*

The complement titer (CH50) was measured using a 50% hemolysis assay with a commercial kit. DHSG-HbV, saline or Coatsome EL-A (a negative-charged liposome) were mixed with serum as indicated ratio (v/v) at 37℃ for 24 hr. The lipid composition (mol%) of Coatsome EL-A was DPPC:CHOL:DPPG=30:40:30. Data are represented as the mean ± SD using serum from five individuals. The CH50 of 100% serum was 38 ± 3.2 U/mL. *$p < 0.05$ vs. saline.

Table 5. Consumption of complement by various types of HbV.

Additive	CH50 (U/mL) (additive:serum)	
	20:80	40:60
Saline	36.4	27.9
DHSG-HbV	37.6	31.4
DPPG-HbV	35.9	28.4
DPPG-HbV(no PEG)	29.9	Under detection limit

The complement titer (CH50) was measured using a 50% hemolysis assay with a commercial kit. DHSG-HbV, DPPG-HbV, DPPG-HbV (no PEGylation) or saline were mixed with serum as indicated ratio (V/V) at 37℃ for 24 hr. The CH50 of 100% serum was 45.1 U/mL.

次に凝固系に及ぼす影響をこれら3種のリポソーム構成成分の異なるHbVを用い，プロトロンビン時間（PT）および活性化部分トロンボプラスチン時間（APTT）について比較した．PTは健常人ヒト血漿との混合比率が上昇するにつれて凝固時間が遅延した．血漿に対し生食の比が20％および60％においては，これら3種のHbVはいずれも生食添加の場合のPT時間を有意に短縮させたが，その差は1秒以内であった．APTT時間に対し，DPPG-HbVとDPPG-HbV（no PEG）は，生食添加の場合のAPPT時間を2秒以内ではあるが有意に短縮させたのに対し，DHSG-HbVはどの血漿比率の場合においても生食の場合と差はみられなかった．

カリクレイン-キニン系に対する影響は，血漿にそれぞれこれら3種のHbVまたは生食を添加し，37℃にて24時間インキュベーション後，血漿中高分子キニノーゲンの分解による低分子化された高分子キニノーゲンの出現をイムノブロッティング法にて検出した．血漿に対し40％あるいは60％にてDPPG-HbVまたはDPPG-HbV（no PEG）を添加させた場合，intactな高分子キニノーゲンが減少し，低分子化された高分子キニノーゲンの増加が顕著に観察される．これに対して，DHSG-HbVではいずれの血漿比率の場合においても生食の場合と差はみられず，DHSG-HbVは血漿中高分子キニノーゲンの分解を引き起こさないことが示された（Fig. 2）．

以上の検討から，陰性荷電のDPPGを構成成分とするHbVではPEG修飾がなければ補体の活性化が惹起されるのに対し，PEG修飾することで補体の活性化が抑えられることが明らかとなった．生理食塩水中でゼータ電位を測定するとDPPG-HbV（no PEG）が-14.5 mV，DPPG-HbVが-3.4 mVであり[30]，表面荷電の影響を裏付けていた．しかしながら，ここに示したごとくDPPG-HbVでは，わずかではあるが，凝固時間，特にAPTT時間の短縮およびカリクレイン-キニン系の活性化を引き起こした．これに対し，DHSG-HbVは，補体系，APPT時間，カリクレイン-キニン系のいずれに対しても影響が無いことが示された．DHSG-HbVのゼータ電位は-2.6 mVでDPPG-

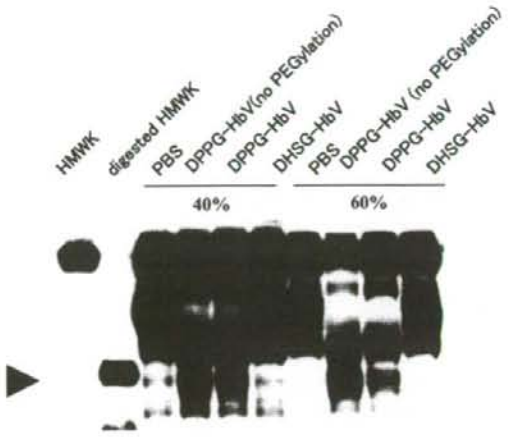

Fig. 2. Activation of kallikrein-kinin cascade by HbVs. HbVs or saline were mixed with plasma at 40％ or 60％ at 37℃ for 24 h. Appearance of digested S-HMWK was detected using western blot analysis as a result of kallikrein activation. Arrowhead (▶) indicates digested S-HMWK.

HbVのそれとほとんど変わらないことから，単に表面荷電だけでなく，構成脂質の親水性頭部（hydrophilic head group）が作用の有無に影響を及ぼすものと考えられる．

いずれにしても，今後の臨床試験に用いられる現行のHbVは，血漿中の補体系に影響を及ぼさないということから，"補体活性化関連偽アレルギー反応"と呼ばれるリポソーム投与直後の心血管系の急性副作用[31]に対する懸念はほとんど無いと推測される．加えて，凝固系やカリクレイン-キニン系への影響も無いことは，ヒト血漿タンパクへの高い適合性を示している．

第1章の血小板に対するHbVの影響に関して，げっ歯類においては血小板上に補体成分C3bが結合するCR1レセプターを有しているが，ヒトを含む霊長類では有さないことを上述した．これに対し，ヒトを含む霊長類では赤血球がCR1レセプターを有しているため，補体活性化に伴って免疫複合体の赤血球への結合が報告されている[32]．そのため，補体活性化を惹起しうる化合物および医薬品のヒト血液を対象としたin vitroにおける適合試験や臨床試験においては，赤血球も相互作用する可能性のある血液構成成分の一つとして重要であり，そのため，赤血球をモニターする意義や結果の解釈にも影響する可能性を考慮しておく必要がある．しかしながら，少なくとも今後の臨床試験に用いられる現行のHbVは，in vitroの評価試験において血漿中の補体系に影響を及ぼさないため，補体成分を介してHbVが赤血球と結合する可能性は少ないと推測される．

おわりに

HbVが臨床応用に向けて，前臨床試験でその有効性，安全性について検討しなければならない事項の中に，免疫系および血液構成成分に対する影響があげられている．ヒト血液細胞およびヒト血漿タンパク系を用いた評価により，止血また炎症反

応にも関与する血小板に対し，HbV自体による活性化作用はみられないこと，またアゴニストによる活性化を亢進するような作用はないことが示された．自然免疫に重要な働きをする好中球の機能（走化能，脱顆粒，活性酸素産生）にも影響を与えなかった．造血機能への影響としては，短期間であれば造血前駆細胞活性を損なうことはないことが示された．またリポソームの特性から補体の活性化が懸念されたが，現行のHbVでは活性化はみられず，また，凝固系，カリクレイン・キニン系にも影響はみられなかった．しかしながらin vitroでは評価できない抗体産生，投与後の補体価の変動，リンパ球の抗原刺激による増殖反応等については現在ラットを用いて検討をおこなっている．本稿にては詳細は割愛したが，それらの影響は，投与直後に一過性の抑制がみられるものの1週間では回復することをみいだしている[28,53]．以上のことから，免疫系，および血液構成成分に対してHbVが高い適合性を持つことを示すことができた．

最近，Natansonら[54]は，ヘモグロビンを基盤とする非セル型の人工酸素運搬体を対象とした無作為化された前臨床試験および臨床試験の中から，一定の基準を満たした16トライアルにおいて，死亡のリスクが30％増，心筋梗塞のリスクが2.7倍増加しているというメタ解析の結果を報告した．この報告に伴って，ヘモグロビンを基盤とする非セル型の人工酸素運搬体の安全性を見直し，リスクの増加の原因を明らかにすべきとの指摘がなされている[55]．このような状況下にあって，我が国だけが開発を進めているセル型の人工酸素運搬体の一つであるHbVに，大きな期待がよせられる．

謝辞

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Pharmacokinetics of single and repeated injection of hemoglobin-vesicles in hemorrhagic shock rat model

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ABSTRACT

Hemoglobin-vesicles (HbV) are liposomal artificial oxygen carriers that may be useful as a resuscitation fluid during hemorrhagic shock (HS). It is well-known that the pharmacokinetic properties of liposome change in response to both pathological conditions and repeated administration. Therefore, we compared the pharmacokinetics of single versus repeated administration of HbV during HS. HS was induced by withdrawal of 40% of total blood volume. The normal (non-HS) and HS_1 group was received an injection of ^{125}I-labeled HbV (^{125}I-HbV). The HS_2 group was resuscitated with non-labeled HbV, and 1 h later, it received an injection of ^{125}I-HbV. The half-life was shorter in HS_1 rats, but it returned to non-HS levels after the second HbV injection. During 12 h after administration of HbV, tissue distribution of HbV was greatest in the HS_1 group; however, the HS_2 group had the greatest tissue distribution at subsequent time points. Excretion into urine, major elimination pathway, did not differ between non-HS and HS_1 rats, but was significantly reduced in the HS_2 group. Furthermore, the half-life of HbV in humans was estimated to be approximately 3–4 days using an allometric equation. This suggests that HbV may be a useful artificial oxygen carrier in HS based on HbV pharmacokinetics.

1. Introduction

Before new drugs are approved for clinical use, they must undergo many types of evaluation, including physicochemical tests, animal studies and clinical trials. Among these assessments, pre-clinical pharmacokinetic studies in various mammalian species are essential, as the results of such studies can be extrapolated to humans, allowing estimation of appropriate dosing regimens in humans. In fact, Kennedy et al. reported that inadequate pharmacokinetic data accounts for approximately 40% of the failed attempts to develop new drug-administration protocols during the clinical-trial stage of drug development [1].

To date, hemoglobin-based artificial oxygen carriers (HBOCs), which include cross-linked [2], polymerized [3] and polymer-conjugated Hbs [4], have been developed to overcome the problems associated with blood transfusion, such as cross-matching, blood-bone infection (human immunodeficiency virus, hepatics virus), and shortage of donated blood. Several of these HBOCs are currently in the final stages of clinical evaluation. Unfortunately, however, some HBOCs cause vasoconstriction, and consequently, hypertension, due to scavenging of nitric oxide (NO) by Hb. This phenomenon may occur because, unlike red blood cells (RBCs), HBOCs lack a cellular structure [5].

In contrast, hemoglobin vesicles (HbVs) are artificial oxygen carriers with a cellular structure similar to that of RBCs: highly concentrated Hb encapsulated in a phospholipid bilayer membrane. Thus, HbV does not cause the adverse effects that are associated with HBOCs [6,7], and HbV can control oxygen release to adjust the amount of allosteric effector [8]. Recently, Sakai et al. reported that the resuscitative effect of HbV is equivalent to that of autologous blood when injected into rats with hemorrhagic shock (HS) induced by withdrawal of 50% of total blood volume [9]. Yamasaki et al. also demonstrated that use of HbV as a cardiopulmonary bypass (CPB) priming solution improved neurologic and neurocognitive outcomes in a rat model of CPB [10].

Despite evidence for the effectiveness of HbV, the pharmacokinetics of HbV has not been well characterized. Thus far, pharmacokinetic studies of HbV have evaluated only changes in serum concentration and organ distribution in normal rats and rabbits administered ^{99m}Tc-labeled hexamethyl-propylenamine oxime (HMPAO) encapsulated in HbV [11]. However, resuscitation after

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massive hemorrhage resulting from clinically relevant causes, such as injury, accidental blood loss or major surgery, has not been studied in detail. Clinical conditions affect the pharmacokinetics of numerous drugs [12,13]. For example, clinical trials have shown that the pharmacokinetics of liposome-encapsulated amphotericin B differ between normal individuals and patients [14,15]. Therefore, clarifying the pharmacokinetics of HbV in animal models of HS should provide useful information, such as dosing regimens, for future clinical applications.

Moreover, clinical use of HbV in patients with HS would require repeated administration of HbV. Recently, PEGylated liposomes were shown to lose their long-circulation half-life after being administered twice to the same animals (referred to as "accelerated blood clearance (ABC) phenomenon") [16]. Therefore, the pharmacokinetics of HbV, which has a liposomal structure, might be altered by repeated administration.

In the present study, we hypothesized that the pharmacokinetics of HbV would be altered by the presence of a pathological condition and by repeated administration. We directly labeled the internal Hb of HbV with iodine to create ^{125}I-labeled HbV (^{125}I-HbV) and subsequently investigated the changes in HbV pharmacokinetics resulting from a pathological condition and repeated administration using a rat model of HS induced by massive hemorrhage.

2. Materials and methods

2.1. Materials and animal

Hb solution, which was purified from outdated donated blood, was provided by the Japanese Red Cross Society (Tokyo, Japan). Pyridoxal 5′-phosphate (PLP) was purchased from Sigma Chemical Co. (St. Louis, MO). 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC), cholesterol, and 1,5-bis-*O*- hexadecyl-*N*-succinyl-L-glutamate (DHSG) were purchased from Nippon Fine Chemical Co. Ltd. (Osaka, Japan). 1,2-distearoyl-*sn*-glycero-3- phosphatidyl- ethanolamine-*N*-PEG (PEG-DSPE) was purchased from NOF Corp. (Tokyo, Japan). Recombinant human serum albumin (rHSA) was given by Nipro Corp. (Osaka, Japan).

Sprague–Dawley (SD) rats (180–210 g) were purchased from Kyudou Co. (Kumamoto, Japan). SD rats were maintained in a temperature-controlled room with a 12-hr dark/light cycle and *ad libitum* access to food and water. All animal experiments were performed according to the guidelines, principles, and procedures for the care and use of laboratory animals of Kumamoto University.

2.2. Preparation of HbV

HbVs were prepared under sterile conditions as previously reported [17]. Briefly, the encapsulated Hb (38 g/dl) contained 14.7 mM of PLP as an allosteric effector to regulate P_{50} to 25–28 Torr. The lipid bilayer was a mixture of DPPC, cholesterol, and DHSG at a molar ratio of 5/5/1, and PEG-DSPE (0.3 mol%). The HbVs were suspended in a physiological salt solution at [Hb] 10 g/dL, filter-sterilized (Dismic, Toyo-Roshi, Tokyo, Japan; pore size, 450 nm), and bubbled with N_2 for storage.

2.3. HbV labeling with ^{125}I (^{125}I-HbV) and preparation of HbV solution

^{125}I-HbV was prepared by incubation of HbV with Na^{125}I (Piscataway, NJ, USA) in an Iodo-Gen (1, 3, 4, 6-tetrachoro-3, 6-diphenylglycoluri) tube for 30 min at room temperature. Thereafter, ^{125}I-HbVs were isolated from free ^{125}I by passage through a PD-10 column (GE Healthcare Bio-Sciences AB). The ^{125}I-HbVs were then filter-sterilized (pore size, 450 nm) to remove aggregates. Over 97% of iodine was bound to internal Hb in HbV. Three different HbV suspensions were prepared: non-radiolabeled HbV and ^{125}I-HbV for HS model rats; and, ^{125}I-HbV for non-HS model rats. All suspensions were mixed with rHSA to adjust the albumin concentration of the vesicle-suspension medium to 5 g/dL. Under these conditions, the colloid osmotic pressure of the suspension is kept constant at approximately 20 mm Hg [17].

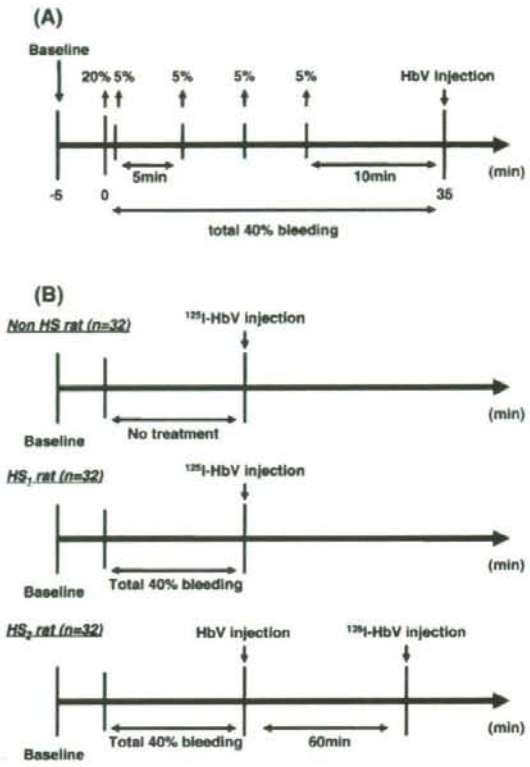

Fig. 1. Scheme illustrating (A) the method of preparing HS model and (B) the pharmacokinetic experimental protocol.

2.4. Preparation of hemorrhagic shock model rats

SD rats were anesthetized with pentobarbital. Subsequently, polyethylene catheters (PE 50 tubing, outer diameter equal to 0.965 mm, and inner diameter equal to 0.58 mm; Becton Dickinson and Co., Tokyo, Japan) containing saline and heparin were introduced into the left femoral artery for infusion and blood withdrawal, and into the right femoral artery to monitor mean arterial pressure (MAP), heart rate (HR) and pressure pulse. HS was induced by removal of 40% of total blood volume (22.4 mL/kg). Systemic blood volume was estimated to be 56 mL/kg [9]. Briefly, blood was removed as follows: constant withdrawal of 20% (1 mL/min); and, removal of 5% (0.5 mL/min) of total blood volume four times over a period of 5 min. After the last withdrawal of blood, animals were allowed to recover for 10 min at room temperature (Fig. 1A).

2.5. Measurement of cardiocirculatory dynamic parameters and plasma clinical chemistry

Ten HS rats were resuscitated by infusion of either isovolemic HbV (1400 mgHb/kg, 1 mL/min; $n=5$) or saline (22.4 mL/kg, 1 mL/min; $n=5$). After resuscitation, the polyethylene catheter of right femoral

artery was removed and the monitoring finished. Blood samples were collected immediately prior to blood withdrawal, after bleeding, and 7 days after resuscitation. Immediately after withdrawal, the pH of all blood samples was measured using a pH meter (HORIBA, Kyoto, Japan). An aliquot of blood was treated with perchloric acid to remove blood proteins for lactate analysis. The remaining blood was centrifuged (3000 g, 5 min) to obtain plasma for analysis of albumin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyltransferase (γ-GTP), amylase, lipase, total-cholesterol, free-cholesterol, HDL-cholesterol, phospholipids, urea nitrogen (BUN), creatinine (CRE) and iron (Fe). All plasma samples were stored at −80 °C prior to analysis by a commercial clinical testing laboratory (SRL, Tokyo, Japan).

2.6. The pharmacokinetic experimental protocol

All rats were given water containing 5 mM sodium iodide (NaI) for the duration of the experiment to avoid specific accumulation in the glandula thyreoidea. Ninety-six male SD rats were anesthetized with pentobarbital and polyethylene catheters were inserted into the left femoral artery. The rats were divided into three groups. Two groups (one-injection group (HS_1), $n=32$; two-injection group (HS_2), $n=32$) of rats underwent experimental HS. Both the non-HS (non-HS group, $n=32$) and HS_1 rats were injected with the ^{125}I-HbV suspension (1400 mg Hb/kg, 1 mL/min). 1 h after the first injection of non-labeled HbV suspension (1400 mg Hb/kg, 1 mL/min), the HS_2 rats received the ^{125}I-HbV suspension (1400 mg Hb/kg, 1 mL/min). The volume of the infused fluid was identical to the volume of blood withdrawn to induce HS (22.4 mL/kg) (Fig. 1B). In each group of rats, five rats were randomly selected to undergo the plasma concentration test. The rats were anesthetized using ether, blood samples were collected at multiple time points after the ^{125}I-HbV injection (3 min, 10 min, 30 min, 1 h, 6 h, 12 h, 24 h, 48 h, 72 h) and the plasma was separated by centrifugation (3000 g, 5 min). Degraded HbVs and free ^{125}I were removed from plasma by centrifugation in 1% bovine serum albumin and 40% trichloroacetic acid (TCA). Three rats in each treatment group ($n=27$/group) were euthanized at each time point (3 min, 30 min, 1 h, 2 h, 6 h, 12 h, 24 h, 48 h, 72 h). The organs were excised (kidney, liver, spleen, lung, heart), rinsed with saline, and weighed. The levels of ^{125}I radiation in the excised organs, urine and feces, collected at fixed intervals (2 h, 6 h, 12 h, 24 h, 48 h, 72 h in each group; urine samples at 2 h and 6 h were not collected in the HS_1 group because of the reduction of renal function) using metabolic cages, were determined using a γ-counter (ARC-5000, Aloka, Tokyo, Japan). A two-compartment model was used to determine the pharmacokinetic parameters after HbV administration. Pharmacokinetic parameters were estimated by curve-fitting using MULTI, a normal least-squares program. Pharmacokinetic analysis was described in the supplementary materials and methods in detail.

2.7. Interspecies scaling of pharmacokinetic parameters

Allometric relationships between various pharmacokinetic parameters (P) and body weight (W) were plotted on a log–log scale. Linear regression of the logarithmic values was calculated using the least-squares method using Eq.(1) to obtain the coefficient (α) and exponent (β) values [18].

$$P = \alpha W^{\beta} \quad (1)$$

To calculate pharmacokinetic parameters, such as V_1 and CL for humans using Eq.(1), the total blood volume of rabbits and humans were assumed to be 5.7% and 7% of body weight, respectively [11]. The CL of rabbits and humans, which were previously reported by Sou et al., were calculated using V_1 and half-life (see supplementary methods Eq. (8)) [11]. The V_1 and CL of mice were 1.75 mL and 0.116 mL/h, respectively (*submitted in separate manuscript*). After prediction of V_1 and CL for humans using Eq.(1), the half-life for human was estimated.

2.8. Data analysis

A two-compartment model was used in the pharmacokinetic analyses after HbV administration. Each parameter was calculated by fitting using MULTI, a normal least-squares program [19]. Data are means ± SD for the indicated number of animals. Dunnett's test was used for comparison of baseline and subsequent values within each group. Significant differences among each group were examined using a Student's *t*-test. A probability value of $p<0.05$ was considered to indicate statistical significance.

3. Results

3.1. Systemic response to hemorrhagic shock

MAP of the SD rats before induction of HS by bleeding was 79.9 ± 4.3 mm Hg; MAP declined significantly to 30.1 ± 1.8 mm Hg after induction of HS. During blood withdrawal to induce HS, MAP remained less than 40 mm Hg (hypotension) for 30 min. After resuscitation with HbV, MAP immediately increased to 81.4 ± 5.6 mm Hg, which was similar to MAP measured at baseline, and apparently HbV did not induce hypertension (Fig. 2A). Average HR before and after hemorrhage was 369 ± 44 and 290 ± 43 beats/min, respectively. However, during the interval between 10-min-post-bleeding and initiation of resuscitation, HR increased slightly (Fig. 2B). Pressure pulse, which reflects cardiac

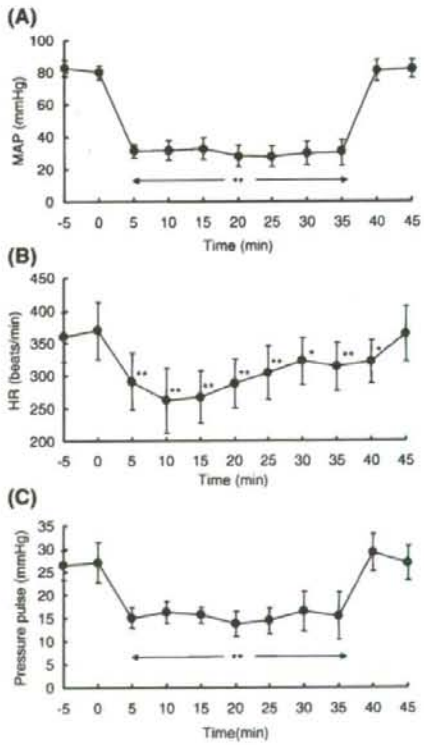

Fig. 2. Change in (A) mean arterial pressure (MAP), (B) heart rate (HR), (C) pressure pulse from −5 min (5 min before bleeding) to 45 min (10 min after resuscitation). Each point represents the mean ± SD ($n=5$). *<0.05 or **<0.01 vs. 0 min.

Table 1
Plasma clinical chemistry analyses after withdrawal of 40% of total blood volume and 7 days after administration of HbV or saline.

	Baseline	After bleeding	7 days after resuscitation	
			HbV	Saline
Albumin, g/dL	3.38 ± 0.13	2.96 ± 0.15	3.34 ± 0.28	3.32 ± 0.26
Phospholipids, mg/dL	124.4 ± 15.4	103.6 ± 12.0	139.2 ± 12.9	129.4 ± 14.2
Total-cholesterol, mg/dL	75.0 ± 11.3	59.4 ± 8.8	80.8 ± 10.3	75.2 ± 9.5
Free-cholesterol, mg/dL	20.2 ± 2.0	16.2 ± 1.9	20.2 ± 2.8	18.8 ± 2.3
BUN, mg/dL	13.1 ± 1.9	13.7 ± 0.6	16.0 ± 1.0	15.6 ± 2.0
Creatinine, mg/dL	0.17 ± 0.01	0.29 ± 0.02[a]	0.19 ± 0.02	0.19 ± 0.01
AST, IU/L	74.0 ± 9.3	65.6 ± 8.7	119.3 ± 28.8[a]	101.8 ± 11.4[b]
ALT, IU/L	42.4 ± 4.7	37.2 ± 2.4	57.0 ± 9.9	56.0 ± 9.7
γ-GTP, IU/L	<2.8	<2.8	<2.8	<2.8
HDL-cholesterol,mg/dL	23.0 ± 3.2	19.2 ± 2.6	28.2 ± 1.5	25.4 ± 2.9
Fe, μg/dL	153.5 ± 36.5	171.4 ± 54.7	148.7 ± 22.5	123.7 ± 66.3
Lipase, U/L	6.8 ± 0.4	6.8 ± 0.8	5.4 ± 0.5	5.8 ± 0.4
Amylase, U/L	669.8 ± 28.5	638.8 ± 34.0	604.0 ± 67.7	624.4 ± 66.0

The values are mean ± S.D. ($n = 3$–5), [a]$p < 0.05$, [b]$p < 0.01$ vs. baseline.

Table 2
Pharmacokinetic parameters of HbV in non-HS rats, HS_1 and HS_2 rats.

	Non-HS	HS_1	HS_2
$t_{1/2\alpha}$, h	5.3 ± 3.9	0.58 ± 0.05[a]	5.0 ± 1.7
$t_{1/2\beta}$, h	30.6 ± 4.0	18.1 ± 3.7[b]	32.4 ± 1.1
C_0, % of dose/mL	9.1 ± 0.2	9.9 ± 0.5	9.8 ± 1.9
V_1, mL	10.9 ± 0.2	10.1 ± 0.5	10.4 ± 2.1
V_2, mL	5.5 ± 1.2	11.9 ± 1.6[b]	5.9 ± 2.0
Vss, mL	16.7 ± 1.1	21.6 ± 2.3[b]	17.0 ± 3.1
ke, $\times 10^{-3}$ min^{-1}	0.70 ± 0.06	1.4 ± 0.1[b]	0.60 ± 0.07
k_{12}, $\times 10^{-3}$ min^{-1}	0.41 ± 0.10	6.6 ± 2.3[b]	7.4 ± 3.5[b]
k_{21}, $\times 10^{-3}$ min^{-1}	0.69 ± 0.23	5.3 ± 1.2[b]	13.9 ± 4.7[b]
AUC, h% of dose/mL	210.3 ± 22.9	126.5 ± 12.8[b]	259.0 ± 48.6
CL, mL/hr	0.47 ± 0.04	0.80 ± 0.08[b]	0.40 ± 0.09
k_{12}/k_{21}	0.60 ± 0.14	1.2 ± 0.1[b]	0.55 ± 0.13

$t_{1/2\alpha}$, the distribution-phase half-life; Vss, the steady-state distribution volumes; k_{12}, the rate constants for the transfer from V_1 to V_2; k_{21}, the rate constants for the transfer from V_2 to V_1.
Values are means ± S.D. ($n = 5$). [a]$p < 0.05$, [b]$p < 0.01$ vs. non-HS rats.

output, was 26.4 ± 3.2 and 15.3 ± 3.1 mm Hg before and after hemorrhage, respectively. After resuscitation, pressure pulse returned to baseline values (Fig. 2C). Acute bleeding resulted in a non-significant decrease in pH from 7.55 ± 0.06 to 7.51 ± 0.05, and a significant increase in lactate from 21.4 ± 4.0 to 62.8 ± 17.5 mg/dL ($p < 0.01$). In addition, skin pallor and hyperpnea developed during hypotension. These results indicate that acute blood withdrawal in rats is an adequate model of clinical HS.

3.2. Plasma clinical chemistry

All rats survived the complete experimental protocol. Table 1 summarizes the clinical chemistry parameters measured in plasma. Immediately after hemorrhage, the concentration of CRE in plasma, which reflects kidney function, was two-fold greater than the concentration at baseline ($p < 0.01$). In contrast, BUN, which is also a marker of kidney function, and other parameters were not affected by hemorrhage. We also performed clinical chemistry analyses in plasma that was collected from HS rats after 7 days of treatment with HbV or saline. Only AST increased significantly, reaching 119.3 ± 28.8 IU/L in the group given HbV ($p < 0.01$ vs. baseline) and 101.8 ± 11.4 IU/L in the group administered saline ($p < 0.05$ vs. baseline). CRE decreased from 0.29 ± 0.02 mg/dL to 0.19 ± 0.02 and 0.19 ± 0.01 mg/dL in the HbV and saline groups, respectively. Although HbV contains many lipids and iron, lipid-related parameters, such as total-cholesterol, free-cholesterol, HDL-cholesterol, phospholipids, and Fe did not differ between the HbV- and saline-treated rats. These results show that neither organ function nor serum parameters were affected by HbV administration, despite the temporary disruption of circulatory function by HS.

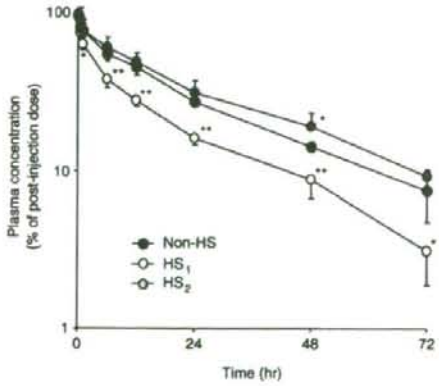

Fig. 3. Relative plasma concentration of ^{125}I-HbV after administration of 1400 mgHb/kg via injection of non-HS, HS_1 or HS_2. Each point represents the mean ± SD ($n = 5$). *< 0.05 or **< 0.01 vs. non-HS rats.

3.3. Plasma concentration

The fate of the ^{125}I-HbV administered to non-HS, HS_1 and HS_2 rats was evaluated by determination of residual TCA-precipitable radioactivity in plasma. Fig. 3 shows the time course of the plasma concentration of ^{125}I-HbV in non-HS, HS_1 and HS_2 rats and Table 2 lists the pharmacokinetic parameters for these groups. The elimination-phase half-life ($t_{1/2\beta}$) of ^{125}I-HbV in HS_1 rats was reduced significantly–by 0.66-fold–compared with the $t_{1/2\beta}$ of ^{125}I-HbV in non-HS rats. In addition, the AUC was decreased, while the elimination rate constant (ke) and clearance (CL) were increased in HS_1 rats compared with non-HS rats. Notably, the initial plasma concentration (C_0) and distribution volume of the central compartment of HbV (V_1) were identical between the two groups, whereas the distribution volume of the peripheral compartment (V_2) in HS_1 rats was nearly two-fold greater than non-HS rats. Interestingly, the plasma concentration of ^{125}I-HbV and pharmacokinetic parameters, except k_{12} and k_{21}, of HS_2 rats were similar to the results observed in non-HS rats. Furthermore, V_1 (approximately 10 mL) was nearly equivalent to blood volume (56 mL/kg).

3.4. Organ distribution of HbV

Fig. 4 shows the time course of organ distribution of ^{125}I-HbV (% of ID) after ^{125}I-HbV administration. Shortly after ^{125}I-HbV administration, the amount of ^{125}I-HbV was significantly greater in the kidney, lung and heart of HS_1 rats compared with non-HS rats; however, there were no differences in distribution of ^{125}I-HbV in other organs between groups. As time passed, HbV was distributed to all observed organs (kidney, liver, spleen, lung and heart) of HS_1 rats, compared with the non-HS rats. In contrast, less ^{125}I-HbV was distributed in the liver, spleen, lung, and heart of the HS_2 rats compared with both non-HS and HS_1 rats. However, past 12 h after administration of HbV, HS_2 rats exhibited the greatest distribution of ^{125}I-HbV in kidney and liver. Furthermore, during the first 12 h after HbV administration, the total amount of ^{125}I-HbV distributed in all observed organs (kidney, liver, spleen, lung and heart) was greatest in HS_1 rats, while after 12 h post-administration of HbV, the greatest amount of total radioactivity was observed in the HS_2 rats.

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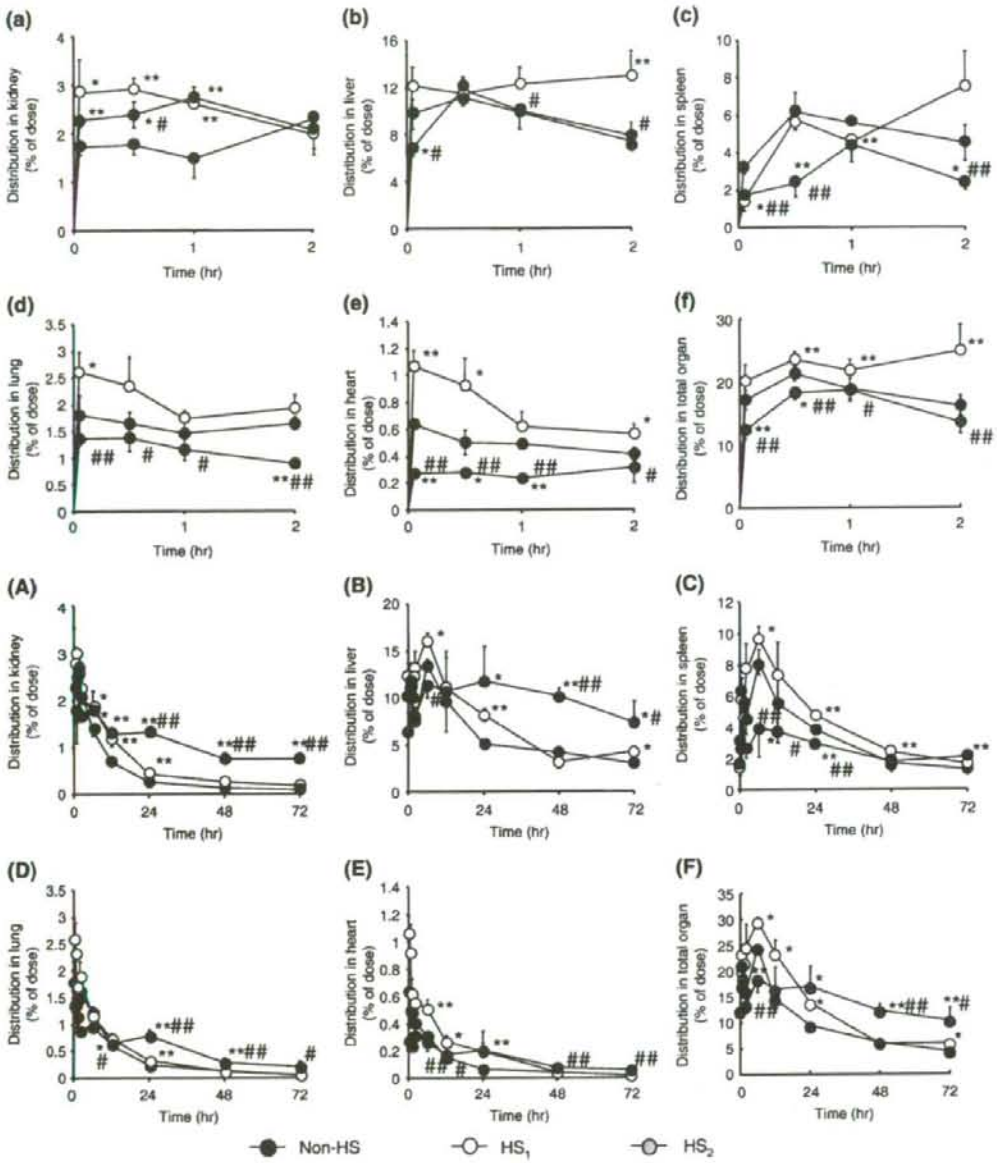

Fig. 4. Distribution of HbV in kidney (a), liver (b), spleen (c), lung (d), heart (e), total organ (f) after injection of non-HS or HS_1 or HS_2 from the time of administration until 2 h post-administration and in kidney (A), liver (B), spleen (C), lung (D), heart (E), total observed organ (the sum of kidney, liver, spleen, lung and heart) (F) from the time of administration until 72 h post-administration. Each point represents the mean ± SD ($n = 3$). *< 0.05 or **< 0.01 vs. non-HS rats. #< 0.05 or ##< 0.01 vs. HS_1.

The tissue distribution of ^{125}I-HbV in non-HS, HS_1 and HS_2 rats was also evaluated based on both percentage of the infused dose/organ weight (ID/g organ) and the tissue-to-plasma partition coefficient (*Kp*), which can be used to compare the kinetics of plasma and tissue HbV concentrations. Table 3 shows the ID/g organ and *Kp* data for the kidney, liver, spleen, lung and heart at 1 h, 12 h and 72 h after administration of HbV. The *Kp* data in spleen became more than 1 (mL/g of tissue), but that in other organ less than 1 (mL/g of tissue) at 1 h and 12 h. At 72 h post-administration of HbV, the % of ID and % ID /g organ values were greatest, the *Kp* value was lowest, and there was an increase in the plasma concentration of HbV in the HS_2 group.

3.5. Urinary and fecal excretion of HbV

To identify the pathways by which HbV is excreted, the amount of HbV in urine and feces was measured in non-HS, HS_1 and HS_2 rats (Fig. 5). We found that HbV was excreted primarily in the urine of all groups (58.9 ± 5.6, 65.5 ± 10.5 and 44.2 ± 2.9% of ID for non-HS, HS_1

Table 3
Dose/gram of organ weight (dose/g) and tissue-to-plasma partition coefficient (Kp) for non-HS rats, HS_1 and HS_2 rats.

		Dose/organ weight (dose/g)			Kp (mL/g of tissue)		
		Non-HS	HS1	HS2	Non-HS	HS1	HS2
1 h							
	Kidney	0.86 ± 0.18	1.32 ± 0.09[b]	1.65 ± 0.14[b,c]	0.14 ± 0.04	0.32 ± 0.02[b]	0.37 ± 0.01[b,c]
	Liver	1.15 ± 0.13	1.09 ± 0.14	0.93 ± 0.44[a]	0.19 ± 0.01	0.26 ± 0.02[b]	0.21 ± 0.03[c]
	Spleen	7.87 ± 0.56	7.11 ± 0.42	9.30 ± 0.44[a,d]	1.30 ± 0.09	1.72 ± 0.10[b]	2.09 ± 0.19[b,c]
	Lung	1.47 ± 0.42	1.48 ± 0.11	1.11 ± 0.16[c]	0.24 ± 0.06	0.36 ± 0.06[a]	0.25 ± 0.03[c]
	Heart	0.81 ± 0.07	0.90 ± 0.17	0.52 ± 0.10[b,c]	0.13 ± 0.01	0.22 ± 0.03[b]	0.12 ± 0.02[d]
12 h							
	Kidney	0.37 ± 0.05	0.64 ± 0.14[a]	0.78 ± 0.09[b]	0.16 ± 0.02	0.29 ± 0.07[a]	0.25 ± 0.03[b]
	Liver	0.87 ± 0.11	1.07 ± 0.31	1.29 ± 0.67	0.39 ± 0.05	0.47 ± 0.02[a]	0.39 ± 0.16
	Spleen	7.23 ± 1.32	10.9 ± 2.69[a]	6.49 ± 1.27[c]	3.19 ± 0.61	4.89 ± 0.72[a]	2.01 ± 0.08[a,d]
	Lung	0.54 ± 0.08	0.71 ± 0.07[a]	0.55 ± 0.10[c]	0.24 ± 0.04	0.32 ± 0.11	0.17 ± 0.01[a,c]
	Heart	0.24 ± 0.07	0.39 ± 0.09[a]	0.30 ± 0.04	0.10 ± 0.03	0.18 ± 0.02[a]	0.09 ± 0.01[d]
72 h							
	Kidney	0.05 ± 0.01	0.08 ± 0.01[b]	0.47 ± 0.15[b,d]	0.21 ± 0.07	0.63 ± 0.01[b]	0.52 ± 0.10[b]
	Liver	0.33 ± 0.07	0.40 ± 0.07	0.75 ± 0.26[a,c]	1.29 ± 0.28	3.12 ± 0.73[b]	0.81 ± 0.15[a,d]
	Spleen	1.52 ± 0.45	1.11 ± 0.16	1.75 ± 0.41[c]	5.74 ± 0.23	7.06 ± 0.11[b]	1.93 ± 0.39[b,d]
	Lung	0.05 ± 0.01	0.05 ± 0.01	0.18 ± 0.07[b,d]	0.18 ± 0.02	0.36 ± 0.09[a]	0.19 ± 0.01[c]
	Heart	0.03 ± 0.01	0.02 ± 0.01	0.09 ± 0.03[b,d]	0.13 ± 0.02	0.19 ± 0.03[a]	0.10 ± 0.01[d]

Values are means ± S.D. ($n = 3$).
[a]$p < 0.05$, [b]$p < 0.01$ vs. non-HS rats. [c]$p < 0.05$. [d]$p < 0.01$ vs. HS_1 rats.

and HS_2 rats, respectively), while radioactivity was virtually undetectable in feces (2.0 ± 0.5, 3.2 ± 1.3 and 2.8 ± 0.5% of ID, for non-HS, HS_1 and HS_2 rats, respectively). Urinary and fecal levels of radioactivity were not significantly different between the non-HS and HS_1 groups. Meanwhile, urinary radioactivity in HS_2 rats was significantly less than that in non-HS and HS_1 rats, and the half-life of HbV was significantly longer in HS_2 rats compared with the other groups.

3.6. Prediction of human pharmacokinetics

As mentioned in the "*Plasma concentration*" section, we found that V_1 was equivalent to arteriovenous blood flow. To predict the CL in humans, we examined the allometric relationship between V_1 and body weight in mice, rats, rabbits and humans. As shown in Fig. 6A, there was a good correlation between V_1 and body weight, with r^2 values of 0.99.

Using the relationship observed for both mice and rats, the CL of HbV in humans was predicted to be approximately 36.6 mL/h. In addition, we applied the rabbit [11] and human CL values for HbV to this relationship. We calculated the human CL (47.2 mL/h) using k_e, which is from Sou *et al.* [11], and our estimated V_1. These values fit our allometric equation well (Fig. 6B). The half-life of HbV in humans was extrapolated to be approximately 96 h.

4. Discussion

HbV is a cellular-type, artificial oxygen carrier that consists of a highly concentrated Hb solution coated with a phospholipid bilayer membrane. Recent findings have demonstrated that HbV restores tissue and plasma oxygenation [9], and improves survival [20] in HS animal models. To exert these pharmacological effects and enhance oxygen-carrying capacity, HbV, or any other RBC substitute, must have a long half-life in circulation ($t_{1/2}$; free Hb and PEG-Hb in rats for 1.5 and 10.9 h, respectively [21,22], while RBC in human for up to 60 days [23]).

In the present study, the elimination-phase half-life ($t_{1/2\beta}$) of ^{125}I-HbV in the HS_1 rats was shorter than that in non-HS rats. This result does not seem to support the therapeutic use of HbV, because the most important determinant of HbV efficacy is HbV-derived Hb concentration in blood. However, the shorter half-life in the HS_1 group may reflect transfer of HbV from arteriovenous blood to the organ capillary beds, thereby normalizing distribution of blood to the organs after bleeding. This scenario would result in an apparent reduction in HbV in arteriovenous circulation. Consistent with this hypothesis, HbV has been shown to both reach microcirculation in critically ischemic peripheral tissue and promote recovery from capillary pathological hemodynamic conditions [24]. In addition, the results of the present study also support our hypothesis: 1) the initial plasma concentration of HbV (C_0) and the amount of HbV eliminated in HS_1 rats were similar to the values observed in non-HS rats, such that the amount of residual HbV in non-HS and HS_1 rats were similar; 2) the time course of HbV distribution in the kidney, liver, spleen, lung and heart in the HS_1 rats was significantly greater than that of the non-HS rats; and, 3) the plasma concentration of ^{125}I-HbV and pharmacokinetic parameters in the HS_2 rats were similar to the values observed in the non-HS rats.

For HbV to keep long circulating retention, the enhanced tissue distribution of HbV is a concern, as it may reflect both increased scavenging of HbV by cells of the mononuclear phagocyte system (MPS), such as Kupffer cells, red pulp zone splenocytes, and mesangial cells [7], and functional changes in MPS after HS. However, alterations in HbV, such as PEG-modification and controlled particle size (250 nm), suppress scavenging by the MPS relative to scavenging of Hb encapsulated in other liposomes [21,25]. Moreover, MPS scavenging of liposomal preparations is influenced by the dose administered [26], while mild hemorrhage does not affect phagocytic activation of Kupffer cells [27]. Therefore, because we used a high dose of HbV in

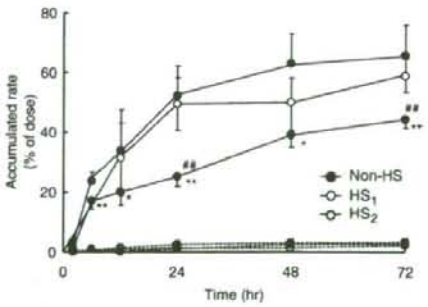

Fig. 5. Time-dependence of urinary and fecal radioactivity after administration of ^{125}I-HbV to non-HS or HS_1 or HS_2. The solid line represents urinary excretion, and the dotted-line represents fecal excretion. Each point represents the mean ± SD ($n = 3$). *< 0.05 or **< 0.01 vs. non-HS rats. ##< 0.01 vs. HS_1.

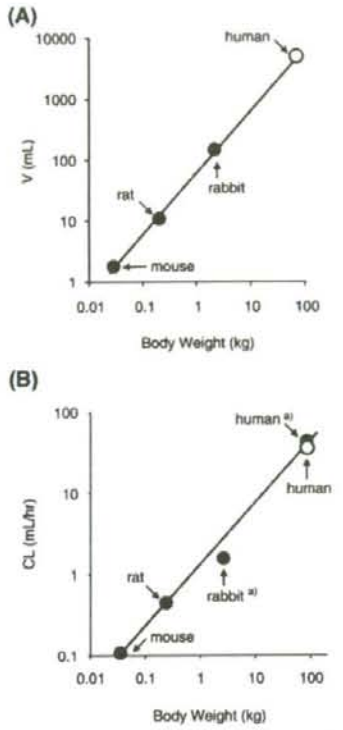

Fig. 6. Allometric relationships between body weight and distribution volume (V_1) (A) and body weight and clearance (CL) (B). The linear regression of the logarithmic values was calculated using the least-squares method (A, $y = 61.48 \times^{1.027}$, $r^2 = 0.99$; B, $y = 1.466 \times^{0.764}$, $r^2 = 0.984$). The extrapolated human values based on a body weight of 70 kg (open circle) are also shown. [a]Data from reference [11] for Rabbit, and we calculated the human CL using k_e, which is from Sou *et al.*[11], and our estimated V_1.

this study, it is unlikely that scavenging of HbV by MPS contributed significantly to the large distribution of HbV to organs during HS. On the contrary, HbV did not induce hypertension just after resuscitation as shown in Fig. 2, which is contrast to the cell-free Hb-based oxygen carriers such as crosslinked Hb and polymerized Hbs [28,29]. This indicates that HbV would not induce vasoconstriction and the blood flow distributes promptly to the capillary bed in all the organs.

In addition, we also were concerned that repeated administration of HbV would shorten circulation half-life by induction of the ABC phenomenon. However, because the half-life of HbV was longer in the HS_2 group compared with the HS_1 group and was not different from the half-life in non-HS rats, the ABC phenomenon did not occur nor did it contribute significantly to the pharmacokinetics of HbV. The dose and dosing interval used in the present study likely prevented induction of the ABC phenomenon. Ishida et al. reported that a low dose and dosing interval of approximately 5 days induced the ABC phenomenon [16]. Furthermore, anti-PEG IgM, which elicits a response by the spleen, plays an essential role in induction of the ABC phenomenon [30,31]. In fact, anti-HbV IgM was detected at 5 days after HbV administration to non-HS rats at a dose of 0.1 mg/kg (low dose), but not detected at 1 h after HbV administration to HS rats at a dose of 1400 mg/kg (data not shown). Therefore, acquisition of immune tolerance and inhibition of anti-PEG IgM production in the present study by use of a high dose and short interval seemed to prevent induction of the ABC phenomenon. In contrast, phagocytic activity, which was studied by measurement of carbon clearance, reportedly was transiently enhanced 3–7 days after administration of HbV at a dose of 2000 mg/kg body weight [32]. Therefore, the altered HbV pharmacokinetics observed in the HS_2 rats might be due to the longer dosing interval. However, in a clinical setting, the dosing interval for treatment of HS would be less than 1 day, so that the effect of phagocytic activity on HbV pharmacokinetics would be minimal.

From the viewpoint of future clinical applications, the allometric prediction of human pharmacokinetics based on data obtained from animal studies–so called, "animal scale-up"–is important for determination of optimal doses and intervals [33]. In the present study, we used an allometric equation that is generally used in animal scale-up to extrapolate the half-life of HbV in humans to be approximately 96 h In contrast, based on half-life and % ID values obtained from pharmacokinetic studies of HbV in rats and rabbits, Sou et al. predicted that the half-life of HbV in healthy humans is approximately 72 h [11]. Differences in predicted HbV half-life may be due to differences in experimental conditions, such as animal species, radiolabeled form and the analytical approach. In fact, the half-life of liposomal doxorubicin (Doxil formulation) in rats and humans is 35 h and 56–90 h, respectively [34]. Furthermore, the half-life of liposomal preparations is 2–3-fold greater in humans than in rats [34]. Therefore, the half-life of HbV in humans seems to be expected 3–4 days from our and previous study [11,34]. These results suggest that HbV functions as an oxygen carrier temporarily until a blood transfusion is available or until autologous blood is recovered after a massive hemorrhage.

5. Conclusion

The pharmacokinetic properties of HbV differed among non-HS, HS_1 and HS_2 rats. To the best of our knowledge, this is the first study to examine changes in the long-term pharmacokinetic characteristics of HbV resulting from a pathological condition and repeated administration using a rat model of HS induced by massive hemorrhage. Our results support that HbV efficacy in HS, which is long enough to meet oxygen-delivery demands until autologous blood volume and oxygen-carrying capacity are restored. Based on the pharmacokinetics of HbV described in this paper, we conclude that HbV might be a useful artificial oxygen carrier during HS.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jconrel.2009.02.009.

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Design and Evaluation of *S*-Nitrosylated Human Serum Albumin as a Novel Anticancer Drug

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ABSTRACT

In recent studies, the cytotoxic activity of NO has been investigated for its potential use in anticancer therapies. Nitrosated human serum albumin (NO-HSA) may act as a reservoir of NO in vivo. However, there are no published reports regarding the effects of NO-HSA on cancer. Therefore, the present study investigated the antitumor activity of NO-HSA. NO-HSA was prepared by incubating HSA, which had been sulfhydrylated using iminothiolane, with isopentyl nitrite (6.64 mol NO/mol HSA). Antitumor activity was examined in vitro using murine colon 26 carcinoma (C26) cells and in vivo using C26 tumor-bearing mice. Exposure to NO-HSA increased the production of reactive oxygen species in C26 cells. Flow cytometric analysis using rhodamine 123 showed that NO-HSA caused mitochondrial depolarization. Activation of caspase-3 and DNA fragmentation were observed in C26 cells after incubation with 100 μM NO-HSA for 24 h, and NO-HSA inhibited the growth of C26 cells in a concentration-dependent manner. The growth of C26 tumors in mice was significantly inhibited by administration of NO-HSA compared with saline and HSA treatment. Immunohistochemical analysis of tumor tissues demonstrated an increase in terminal deoxynucleotidyl transferase dUTP nick-end labeling-positive cells in NO-HSA-treated mice, suggesting that inhibition of tumor growth by NO-HSA was mediated through induction of apoptosis. Biochemical parameters (such as serum creatinine, blood urea nitrogen, aspartate aminotransferase, and alanine aminotransferase) showed no significant differences among the three treatment groups, indicating that NO-HSA did not cause hepatic or renal damage. These results suggest that NO-HSA has the potential for chemopreventive and/or chemotherapeutic activity with few side effects.

Although cancer primarily arises from disorders of cell proliferation, it also may arise from disruptions in programmed cell death signaling pathways, resulting in decreased apoptosis of cancerous cells (Okada and Mak, 2004). Therefore, induction of apoptosis in neoplastic cells is a very effective therapy for tumor eradication (Meng et al., 2006). However, this type of chemotherapy often has negative side effects, such as transient cell cycle arrest, senescence, and autophagy. Drug delivery systems that facilitate selective apoptosis of neoplastic cells have been suggested as a way of overcoming this problem (Kaufmann and Gores, 2000; Kondo et al., 2005).

NO is a unique diffusible molecular messenger that occupies a central role in mammalian pathophysiology (Brune et al., 1998). Its multiple actions include vascular smooth muscle relaxation (Moncada et al., 1986; Ignarro, 1989), inhibition of platelet aggregation (Azuma et al., 1986), effects on neurotransmission (Garthwaite, 1991), and regulation of immune function (Marletta et al., 1988). Alternatively, under some circumstances, NO is cytotoxic (Laval and Wink, 1994). NO causes cellular iron losses and inhibits DNA synthesis, mitochondrial respiration, and aconitase activity in L10 hepatoma cells (Hibbs et al., 1988). In addition, NO reacts with

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ABBREVIATIONS: NSAID, nonsteroidal anti-inflammatory drug; ASA, aspirin; HSA, human serum albumin; DTPA, diethylenetriaminepentaacetic acid; HBSS, Hanks' balanced salt solution; rHSA, recombinant human serum albumin; PAGE, polyacrylamide gel electrophoresis; C26, murine colon 26 carcinoma; ROS, reactive oxygen species; PBS, phosphate-buffered saline; CM-H_2DCFDA, 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; Cr, serum creatinine; BUN, blood urea nitrogen; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; BSA, bovine serum albumin; GSNO, *S*-nitrosoglutathione; R410C, genetic variant of human serum albumin mutated at position 410.

superoxide anion (which is produced by activated macrophages and other cells), to form peroxynitrite. This by-product of NO is a potent chemical oxidant, which alters protein function and damages DNA (Beckman and Crow, 1993). These effects are part of the nonspecific host defense, which facilitates killing of tumor cells and intracellular pathogens. In addition, the cytotoxicity arising from long-lasting NO generation has been attributed to induction of apoptosis (Brune et al., 1998).

In recent studies, the cytotoxic activity of NO has been studied to assess its therapeutic potential in cancer treatment. NO-donating nonsteroidal anti-inflammatory drugs (NSAIDs), especially NO-aspirin (NO-ASA), have been investigated as promising chemopreventive agents (Williams et al., 2001; Kashfi et al., 2002; Fabbri et al., 2005). NO-ASA consists of traditional ASA to which an NO-releasing moiety is bound via a spacer. This agent induces oxidative stress by increasing intracellular peroxide and O_2^-, thereby inducing apoptosis via activation of the intrinsic apoptosis pathway (Gao et al., 2005). JS-K is a prodrug designed to release NO after reacting with glutathione transferase, which induces double-stranded DNA breaks, activates DNA damage response pathways, and induces apoptosis in human multiple myeloma cells both in vitro and in vivo (Kiziltepe et al., 2007).

Human serum albumin (HSA) is an abundant circulating protein, and the nitrosated form serves as a reservoir of NO (Stamler et al., 1992). Therefore, NO-HSA is an NO donor that is currently being investigated for its potential therapeutic applications. For example, administration of NO-HSA to animals with ischemia-reperfusion injury minimizes the tissue damage that occurs after reperfusion (Semsroth et al., 2005). In a balloon-injured rabbit femoral artery model, locally delivered NO-HSA preferentially binds to sites of vessel injury and inhibits both platelet accumulation and the subsequent development of neointimal hyperplasia (Marks et al., 1995). NO-HSA also shows potent antibacterial activity and inhibits the proliferation of cultured human vascular smooth muscle cells (Ishima et al., 2007). However, there are no reports describing the effects of NO-HSA on cancer.

Accordingly, the present study evaluated the antitumor activity of NO bound to HSA (NO-HSA) via an *S*-nitrosothiol linkage using iminothiolane as a spacer. The molecular events related to induction of apoptosis by NO-HSA were studied in vitro, and the antitumor activity of NO-HSA was studied in vivo using a murine model of C26 colon carcinoma.

Materials and Methods

Chemicals. Traut's reagent (2-imminothiolane) was purchased from Pierce Chemical (Rockford, IL). Isopentyl nitrite, diethylenetriaminepentaacetic acid (DTPA), and Cell Counting Kit-8 (WST-8) were purchased from Wako Pure Chemicals (Osaka, Japan). RPMI 1640 medium, Hanks' balanced salt solution (HBSS), and RNase A were obtained from Sigma-Aldrich (St. Louis, MO). Proteinase K was obtained from Roche Applied Science (Indianapolis, IN). All other reagents used were of the highest grade available from commercial sources.

Expression and Purification of Recombinant HSA. rHSA was produced using a yeast expression system as described previously (Matsushita et al., 2004). In brief, for constructing the HSA expression vector pPIC9-HSA, native HSA coding region was incorporated into the methanol-inducible pPIC9 vector (Invitrogen, Carlsbad, CA). The resulting vector was introduced into the yeast species *Pichia pastoris* (strain GS115) to express rHSA. Secreted rHSA was isolated from the growth medium by a combination of precipitation with 60% (w/v) $(NH_4)_2SO_4$ and purification on a Blue Sepharose CL-6B column (GE Healthcare, Little Chalfont, Buckinghamshire, UK) followed by Phenyl HP column (GE Healthcare). Isolated protein was defatted by using the charcoal procedure described by Chen (1967), deionized, freeze-dried, and then stored at −20°C until use. The resulting rHSA (treated with dithiothreitol) exhibited a single band on SDS-PAGE. Density analysis of protein bands stained with Coomassie Brilliant Blue showed that its purity was more than 97%.

Synthesis of NO-HSA. Terminal sulfhydryl groups were added to the HSA molecule by incubating 0.15 mM rHSA with 3 mM Traut's reagent in 100 mM potassium phosphate buffer containing 0.5 mM DTPA, pH 7.8, for 1 h at room temperature. The resultant modified rHSA then was *S*-nitrosylated by 3-h incubation with 15 mM isopentyl nitrite at room temperature (Fig. 1). The resulting NO-HSA was concentrated, exchanged with saline using a PelliconXL filtration device (Millipore Corporation, Billerica, MA), and the final concentration adjusted to 2 mM NO-HSA. The sample was stored at −80°C until use.

Determination of S-Nitrosylation Efficiency. The amount of the *S*-nitroso moieties of NO-HSA was quantified using a 96-well plate. First, 20-μl aliquots of NO-HSA solution and $NaNO_2$ (standard) were incubated with 0.2 ml of 10 mM sodium acetate buffer, pH 5.5, containing 100 mM NaCl, 0.5 mM DTPA, 0.015% *N*-1-naphthylsthylene-diamide and 0.15% sulfanilamide with or without 0.09 mM $HgCl_2$, for 30 min at room temperature. Then, the absorbance was measured at 540 nm. The number of moles of NO per mole of HSA was obtained by subtracting the values in the absence of $HgCl_2$ from values in the presence of $HgCl_2$; the value thus obtained, was 6.64 ± 0.54 mol NO/mol HSA.

Cellular Experiments with C26 Cells. C26 cells, which were donated by the Institute of Development, Aging and Cancer, at Tohoku University (Sendai, Miyagi, Japan), were cultured at 37°C in RPMI 1640 medium containing 10% fetal calf serum, 100 U/ml penicillin, and 10 μg/ml streptomycin (medium A). Trypsin (0.25%) EDTA solution was used to detach the cells from the culture flask for plating and passing the cells. All cell culture experiments were performed at 37°C in a humidified atmosphere of 5% CO_2 in air.

For detection of reactive oxygen species (ROS), C26 cells (1.0×10^4 cells/well) were cultured in 96-well plates in medium A for 12 h, they were washed twice with PBS, and then they were incubated for an additional 30 min in HBSS containing 5 μM 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA) (Invitrogen, Carlsbad, CA). After washing twice with HBSS, the cells were cultured in HBSS containing 5% fetal calf serum for 15 min followed by the addition of PBS, 50 μM HSA, or 50 μM NO-HSA. After incubation, fluorescence was measured using a plate reader (excitation wavelength, 485 nm; emission wavelength, 535 nm). The change in fluorescence was calculated by subtracting the fluorescence at 0 h from the fluorescence measured at the indicated times. The fluorescence intensities of cells incubated with PBS, 50 μM HSA, and 50 μM NO-HSA at 0 h were 201.3, 166.1, and 181.3, respectively.

Changes in the mitochondrial membrane potential of C26 cells

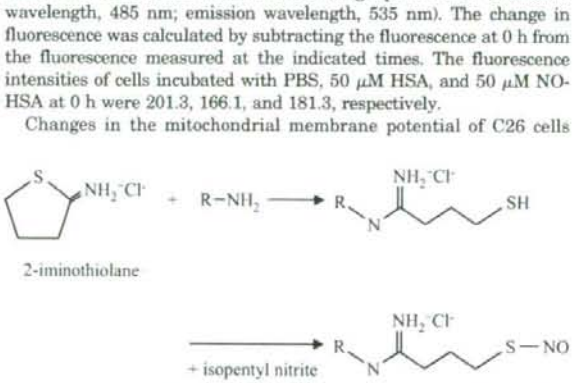

Fig. 1. The scheme for the reaction of 2-iminothiolane with primary amines followed by *S*-nitrosylation.