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（武 岡 真 司）

別刷 50部

講　座

人工赤血球・人工血小板の開発の現状

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はじめに

筆者の所属する研究グループ（早稲田大学理工学総合研究センター）は，慶應義塾大学医学部と共同して厚生労働省科学研究，医薬品・医療機器などレギュラトリーサイエンス総合研究事業，H16-医薬-067，069，071にて，人工赤血球と人工血小板の研究を推進している．人工血液全体の現状に関しては，厚生労働省科学研究の研究代表者小林紘一教授による本誌「講座」に詳しい[1]．本「講座」では，これらの厚生労働科学研究の研究成果の一部も含めて報告する．人工赤血球は，リン脂質の二分子膜小胞体（リポソーム）に酸素を酸素分圧に応じて吸収・脱着する分子（ヘモグロビン）を内包させた酸素運搬体であり，これが血中に長く留まって安全，安定に酸素運搬機能を発現し続ける．それに対して人工血小板は，血管損傷部位や活性化した血小板のみを認識する分子をリポソームやアルブミン重合体に担持した微粒子であり，これが血中に長く留まって血管損傷部位に特異的に粘着して止血機能を発現する．筆者らは理工学の立場から人工赤血球や血小板の材料となる担体の設計，製造，物性評価を行ってきた．担体には，適当な血液適合性や血中滞留性が求められるが，分解性や代謝物の低毒性も重要な検討項目である．現在担体としてリン脂質分子の集合体（リポソーム）や遺伝子組み換えヒトタンパク質の複合体や重合体を選択して用いている．

キーワード：人工赤血球，人工血小板，ヘモグロビン

Seminar
Current Development of Artificial Red Blood Cells and Artificial Platelets
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1. ヘモグロビン小胞体の構造

人工赤血球としてパーフルオロカーボン乳剤や修飾ヘモグロビンなどが検討され臨床使用されてきたが，機能や安全性の観点から満足できるものではなかった．筆者の所属するグループで開発を進めている，高濃度ヘモグロビンをリポソームの内水相に内包させた，赤血球と類似構造のヘモグロビン小胞体(図❶)は，最も安全度と機能が高いため早期の臨床試験着手が期待されている[2]．現段階では期限の切れた献血血液由来のヘモグロビンの有効利用が進められているが，将来的には遺伝子組換えヒトヘモグロビンが利用されるであろう．赤血球からヘモグロビンを精製する際に，血液型を決める型物質やヘモグロビン以外のタンパク質，ウイルスや菌（もし含まれたとしても）を加熱やフィルター処理で除去されている．生理活性なヘモグロビンを安定なリン脂質膜で包むことによって，ヘモグロビンに由来する副作用（血管収縮や腎毒性，神経毒など）を回避できる．ヘモグロビン小胞体は生理食塩液に分散され，脱酸素状態で容器に密封されているため，室温で2年間の液状保存（赤血球製剤では採血後3週間の冷蔵保存）が保証されており，乾燥粉末ではさらに長期間の保存が可能である．製剤のヘモグロビン濃度

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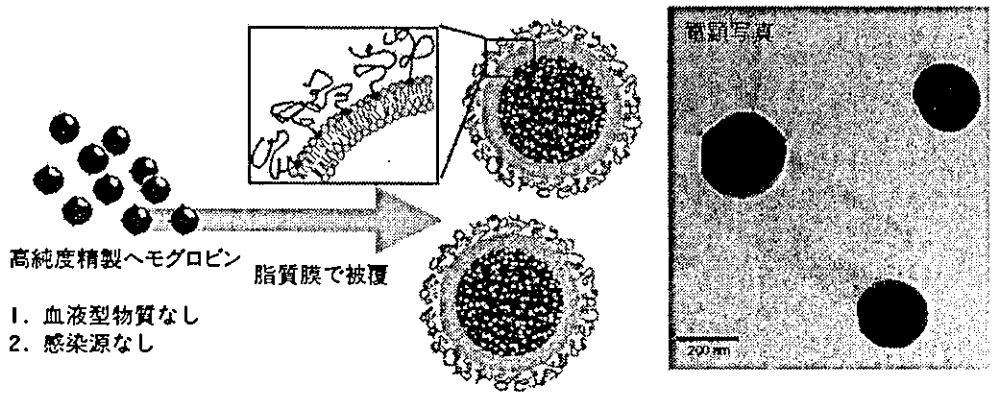

図❶　ヘモグロビン小胞体の構造と模式図ならびに透過型電子顕微鏡写真．

は10 g/dlであり，ヒト血液の値（11～15 g/dl）と比較して遜色ない．また，ヘモグロビン分子が封入されているため製剤の膠質浸透圧はほとんどゼロである．したがって，膠質浸透圧の調節が必要となる場合にはアルブミン（リコンビナント）や多糖類などのコロイド製剤と併用となる．図❶の電子顕微鏡写真では，ヘモグロビンの鉄が染色されており，数多くのヘモグロビンが脂質分子膜で包まれた小胞体構造と，粒子径が約250 nmに厳密に調節されていることがわかる．これは，赤血球の約1/30程度であるので，梗塞部位の透過など赤血球にはない機能が期待できる．酸素親和度はアロステリック因子，ピリドキサール5′-リン酸の共封入により適当値に調節されている．脂質類の成分組成~~と含量~~には，ヘモグロビンのカプセル化効率，常温で2年間液状保存できる安定性，血流中での溶血の回避と適度な血中滞留時間（人では3日程度の半減期予測），血小板や補体の活性化の回避など，に対する工夫が施されている．製造面でも分子集合技術を利用した粒子径の厳密な制御と高濃度ヘモグロビンの内包など，従来の小胞体における課題が解決できている[3]．

2. 動物試験による機能と安全性の評価

現在までに結果が得られているヘモグロビン小胞体に関する評価試験成績を簡単に紹介する．主にラットやハムスターを用いた試験であるが，基本的な安全性と酸素輸送効果は十分確認できている．また，現在霊長類を用いた安全性試験が進行している．酸素運搬効果を確認する試験として，ラット全血液量の90％をアルブミン単独で交換した場合には70％交換あたりから血圧と腎皮質酸素分圧の低下が顕著となって死亡したが，ヘモグロビン小胞体をアルブミン溶液に分散させた系で90％交換した場合には血圧，腎皮質酸素分圧ともに維持された[4]．ヘモグロビン小胞体のアルブミン分散液によるハムスター80％交換輸血試験では，非侵襲に測定した皮下微小循環系の組織酸素分圧は交換前の60～70％に低下するものの，対照アルブミン投与群よりも5倍以上の値が維持されていた[5]．さらにNZW兎を用いた検討では，人工呼吸下，脱血し平均血圧を30～35 mmHgに低下させた後，ヘモグロビン小胞体分散液を投与し，組織酸素分圧の多点測定を実施，とくに脳と腎臓でヘモグロビン小胞体が有意な回復効果を発揮することを明らかにしている[6]．中型動物を用いた実験としてビーグル犬（約7 kg）を用い人工呼吸下，脾臓摘出後アルブミンで75％血液希釈後さらに30％脱血し，30分経過後に人工赤血球を投与し，循環動態，血液ガス組成，組織酸素分圧，組織酸素化度，心拍出量，血中酸素濃度の回復が確認されている[7]．

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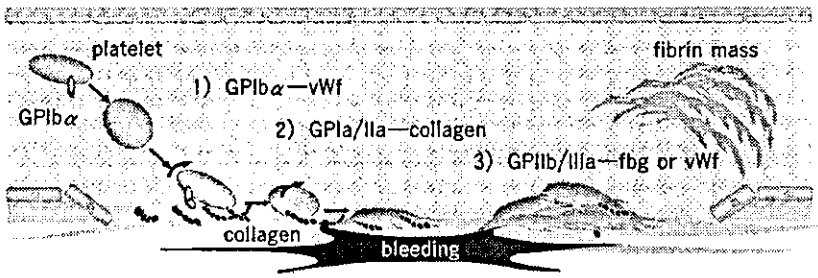
安全面では，血管弛緩因子である一酸化窒素や一酸化炭素が関与してヘモグロビンに認められる抵抗血管の収縮と血圧亢進は，ヘモグロビン小胞体では認められなかった[8,9]．これは，ヘモグロビン小胞体の大きさが寄与しているものと思われる．ヘモグロビン小胞体の血中滞留時間は，ラット，ラビット，カニクイザルからヒトへ類推すると，3日程度の半減期が見積もられ，緊急時の単回投与では十分とされる．ラットでは，血中半減期が1～1.5日であるので，脾臓や肝臓の病理組織学的所見では，投与1日後には脾臓や肝臓に貪食されていたヘモグロビン小胞体は3日後には激減し，投与7日以内にはほとんど消失していた[10]．また，ラットでの単回交換投与（循環血液量の40%交換），反復負荷投与（10 ml/kg/day，14日間）による血液生化学試験（30項目）や病理試験での詳細から，ヘモグロビン小胞体成分である，脂質分解に関わるリパーゼの亢進，コレステロール値の上昇，鉄の沈着，細網内皮系の肥大が一過性に認められた以外に変動を認めていない[10,11]．その他免疫系，凝固系への影響も認められておらず，大量出血時の緊急対応では十分なる機能を発現するものと期待されている．

上述のような効果と安全性の高い人工酸素運搬体では，輸血の代替以外にさまざまな適応が検討されている．たとえば体外循環回路補充液としての利用の検討では，ラット体外循環モデルの作成のため小型人工心肺を試作し，ヘモグロビン小胞体分散液を充填液として使用，血液交換率が50%以上になる条件で灌流させた後，灌流回路中のヘモグロビン小胞体を分離除去して赤血球を回収して投与し，長期生存できることを確認している[12]．虚血性疾患の治療への利用においても，虚血再灌流実験などで小粒径のヘモグロビン小胞体の効果を実証する *in vivo* 実験が進められている．*in vitro* では，微小血管モデル内を流動するヘモグロビン小胞体の酸素放出挙動の解析から，虚血領域酸素化の機序解明を進められている[13,14]．人工酸素運搬体は，腫瘍組織酸素化にも有効であることを実証し，新しい適応の可能性を提示された[15]．

3. 人工血小板の開発の考え方

血小板は出血部位に対し特異的粘着，伸展，凝集，放出，血液凝固系の活性化などの複雑な機能をもち，これらのすべてを兼備した血小板代替物の構築は事実上不可能であろう．しかし，血小板の粘着と凝集に着目して，これらの機能を付与させた担体の投与によっても，少数残存する血小板の機能補助ができるものと考えられる．筆者らは慶應義塾大学医学部内科 池田康夫教授のグループ



図❷ 血小板の止血機構．
一次止血（血小板血栓）
1) 接着（tethering → rolling）GPIbα—vWf
2) 粘着（adhesion）GPIa/IIa—collagen
3) 凝集（aggregation）GPIIb/IIIa—fbg or vWf
二次止血（フィブリン血栓）

とともに，血小板膜タンパク質の一部の遺伝子組換え体や合成オリゴペプチドを担持させた微粒子を作成し，これらが血小板を巻き込んで出血部位へ集積することによって止血能が発現されることを期待して，研究を進めている[16]．

血小板による止血は，高ずり速度の血流と低ずり速度の血流では機構が異なる．図❷に示したように，高ずり速度の出血に対する血小板の止血は，出血部位に露出する血管内皮下組織であるコラーゲンに結合したフォンビルブランド因子(vWf) に対して，血小板が認識して接着して転がることから始まる．*in vitro* 観測で抗GPIIb/IIIa抗体を添加してGPIIb/IIIaの機能を阻害した血小板では，vWf固定化基板上を流動方向に沿って転がる現象がみられ，この認識能は血小板表面のGPIb/V/IX複合体のGPIbα部が担っている[17]．次に血小板表面のGPIaIIa（$\alpha_2\beta_1$インテグリン）やGPVIが直接コラーゲンと相互作用して血小板は粘着し，そこで活性化されると血小板は伸展して顆粒を放出するが，最も重要な過程はGPIIb/IIIa（$\alpha_{IIb}\beta_3$インテグリン）が活性体となる現象である．フィブリノーゲンは，この活性体を認識して血小板間を架橋し凝集体を形成して一次止血を担う．引き続く凝固系の誘導によるフィブリン塊の形成（二次止血）によって止血が完成する．そこで，高ずり速度の血流下でvWfを介してコラーゲンを認識するGPIbα，低ずり速度でコラーゲンを直接認識するGPIaIIa，活性化血小板上のGPIIb/IIIaを認識するフィブリノーゲン（Fbg）やその認識部位であるペプチドを候補とした．

4. 人工血小板の研究動向

採血液に抗GPIIb/IIIa抗体を添加してGPIIb/IIIaを阻害すると，血小板表面のGPIbαとの相互作用によってvWf固定化基板上を流動方向に沿って転がる現象がみられる．そして，rGPIbαを担持させたリン脂質小胞体でも血小板と同様にvWf基板上を転がることが確認された[18]．転がる小胞体の数はずり速度が高くなるほど多くなり，rGPIbαの特性が確認できた．また，その転がり速度は小胞体を構成する膜の柔軟性と相関した[19]．すなわち“柔らかい”小胞体では転がり速度は低くなり，“硬い”小胞体では転がり速度は高くなった．他方，アルブミン重合体は，内部が充填された無定形な綿雪のような形態をとっており，出血部位での充填効果が期待できる．表面にrGPIbαを結合させたところ，小胞体のようなvWf基板を転がる挙動は全く認められず，高ずり速度下でも粘着する挙動が認められた．ラテックスビーズにrGPIbαを結合させた系でも粘着することから，担体が重合体である場合と膜構造をもつ場合ではrGPIbα機能の発現の仕方が異なることが示唆された[20]．

他方，主に低ずり速度の血流下でコラーゲンに直接結合する血小板膜タンパク質の遺伝子組換え体（rGPIaIIa）を結合させた小胞体は，コラーゲン基板を特異的に認識して粘着（停止）することが西谷ら[21]によって確認された．また，ずり速度が高くなるにつれ粘着数は減少するが，rGPIbαとrGPIaIIaともに担持させた小胞体では，低ずり速度から高ずり速度までコラーゲン基板を粘着できる系が構築されている[22]．

さらに減少した残存血小板の凝集を補助するために，粘着して活性化した血小板同士を架橋するフィブリノーゲンを結合させたアルブミン重合体も検討した[23]．活性化血小板の固定化基板を作成し，フィブリノーゲン結合アルブミン重合体を流動させたところ基板上に一様に粘着し，抗GPIIb/IIIa抗体を添加した系やアルブミン重合体のみの系では粘着が抑制された．血小板数が正常値の1/5程度に調節された血小板減少モデル血液に添加するフィブリノーゲン結合アルブミン重合体の濃度増大とともに流動血小板の粘着数が増大したことから，フィブリノーゲン結合アルブミン重合体は血小板粘着増強効果を有する微粒子であることが示唆された．しかし，フィブリノーゲンは不安定であり，しかも現状ではヒト血液由来となるため，Fbgのγ鎖C末端アミノ酸序列（H12：HHLGGAKQAGDV）を結合させた系

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を用いた研究を重点的に進めている[24]．H 12結合アルブミン重合体を血小板減少血液（[血小板]＝2.0×10⁴/μl）に添加しコラーゲン基板上に流動させたところ，粘着血小板の占有率が増加し，そこにH 12結合アルブミン重合体が巻き込まれていたのでH 12-polyAlbは血小板凝集を補強する効果があると考えられた．

筆者ら[25]は抗がん剤であるブスルファン投与の副作用によって血小板が減少したラットを用いて *in vivo* 効果試験を行っている．血小板数が正常値の1/5程度まで減少した状態のラットに対して，セボフルラン麻酔後試料を尾静脈投与した．試料投与5分経過後，尾先端から1 cmの部位にクイックヒール（ベクトン・ディッキンソン社製）を用いて傷（長さ2.5 mm，深さ1 mm）をつけ，尾先端を生理食塩水液に浸して止血時間を計測した．また，試料投与5分前，投与30分後に採血し，各血球変動を観察した．コントロールとして生理食塩液を投与した血小板減少症モデルラット群（[血小板]＝19.8±2.8/μl）の出血時間は609±153秒であり，正常ラット群（[血小板]＝80.9±8.6/μl）の出血時間（178±56秒）と比較して約3.4倍延長した．H 12結合していないアルブミン重合体を40 mg/kg投与したところ，出血時間は短縮し（184±69秒），投与量の減少に伴いその効果は減少した．したがって，アルブミン重合体自体でも止血効果を有することが示唆された．そこで，出血時間に影響しないアルブミン重合体の投与量（4 mg/kg）でH 12-アルブミン重合体の止血能を検討した．H 12-polyAlbの投与では，出血時間352±73秒となり出血時間は半分に短縮したが，逆配列H 12を結合させたアルブミン重合体では出血時間を短縮させないので，H 12の効果が確認された．さらに検体投与5分前，投与30分後に採血し，各血球変動を観察したところ，各検体投与前後における血球変動は生じていないことから，H 12-アルブミン重合体は血液適合性の高い微粒子系と思われた．さらにポリエチレングリコールにてアルブミン重合体を表面修飾し，一部のポリエチレングリコール鎖末端にH 12を結合させた系では，投与後3時間後に同様の試験を行っても止血効果が持続していることが確認された．

他方，rGPIaIIaを担持させたアルブミン重合体ではX線照射で血小板数を正常値の1/5程度に減少させたマウスに投与したところ，コントロール群の出血時間（730±198秒）と比較して，投与量依存的に出血時間の短縮が認められた（たとえば2.4×10¹¹particles/kgでは出血時間は337±46秒）[26]．

現在，GPIbαを結合させたアルブミン重合体やリポソームの系で *in vivo* 試験が進行中であるが，予想どおりの結果が得られつつあるので，今後はこれらの混合系における最適化を目指している．

おわりに

人工赤血球は臨床試験を目指して，企業がGLP製造を行う段階に入っている．また，人工血小板の研究は，動物試験での効果と安全性を多角的に確認している段階にある．これらの製剤はいずれもわが国が最先端にあるため，有効性や安全性の試験項目や方法の設定やガイドラインの作成に対して迅速で慎重なる検討が必要である．そのためには，産官学の共同体制での研究や協議の場として，学会（たとえば日本血液代替物学会や関連学会）の果たす役割と責任も大きいと思われる．産業においては，ナノバイオロジクス領域における具体的な成果として，わが国の近未来医療への貢献はもとより，安全な血液が不足している多くの国に対しても大きな国際貢献と成り得る．ぜひとも長期的そして全人類的な視野に立った開発を期待したい．

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Design and Modification of Nanoparticles for Blood Substitutes

SHINJI TAKEOKA

Key words. Liposome, Nanoparticle, Hemoglobin, Red-blood-cell substitute, Platelet substitute

Introduction

We have been developing the technology of stabilized and functionalized nanoparticles such as liposomes for 20 years. When phospholipids and cholesterol are dispersed into an aqueous solution, they spontaneously assemble to form vesicles with a bimolecular (bilayer) membrane. There are many parameters, such as size, size distribution, lamellarity (the number of bilayer membranes), membrane fluidity, surface charge, surface modification, membrane permeability, that characterize liposomes. They can be adjusted as need dictates to allow for changing encapsulation of functional molecules, release triggered by external stimuli, conjugation of functional sites on the surface, rolling or adhesion properties of liposomes, and control of blood circulation time. On the other hand, we have to consider their physical and chemical stability during storage or blood circulation. Surface modification with polyoxyethylene (POE) chains is one of the most effective ways to impart such stabilization.

In this chapter, I introduce two examples of nanoparticle application; one is a liposome encapsulating concentrated hemoglobin (Hb-vesicle) for a red-blood-cell substitute, and the other is a liposome bearing recognition proteins or peptides on the surface and used as a platelet substitute. The microcirculation, pharmacokinetics, and histopathological change were studied in relation to the characteristics of the particles as well as their oxygen-binding and releasing properties. In the case of the platelet substitutes, nanoparticles

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bearing receptor proteins of the platelet surface recognize the collagen surface under shear rates. The rolling and adhesion properties will be discussed depending on the stiffness or membrane fluidity of the particles.

Present Status of the Development of Red-Blood-Cell Substitutes [1,2]

Hb-vesicles that encapsulate concentrated hemoglobin with a phospholipids bilayer membrane have a similar structure to red blood cells, and are expected to be used soon in clinical tests because the degree of safety and efficacy are considered to be high. Although effective use of the hemoglobin from donated and expired blood should be promoted at the present stage, recombinant human hemoglobin will be used in the future. During hemoglobin purification from red blood cells, stroma including the glycoproteins which determine a blood type, proteins other than hemoglobin, and the viruses are removed by heating or filter processing. By encapsulating hemoglobin with a stabilized phospholipids membrane with POE-lipid, liquid-state preservation for 2 years is guaranteed at room temperature under nitrogen atmosphere [3], and with dry powder, further prolonged preservation is possible. These points are advantages for an artificial oxygen carrier.

The design of the red-blood-cell substitutes (POE-modified hemoglobin vesicles) are summarized in Fig. 1. Hb-vesicles are dispersed into a saline solution and enclosed with the bottle in a state of deoxidization. The hemoglobin concentration is 10 g/dl and is close to that of human blood. Moreover, because hemoglobin molecules are encapsulated, the colloid osmotic pressure of the solution is zero. Therefore, when regulation of colloid osmotic pressure

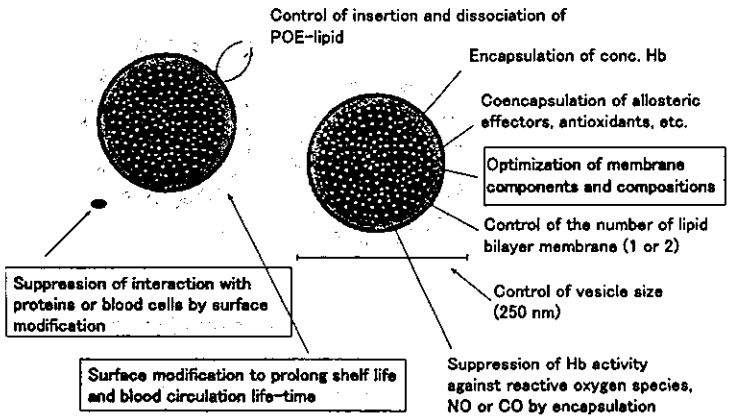

FIG. 1. Design of POE-modified hemoglobin vesicles (Hb-vesicles) as red-blood-cell substitutes

is needed, a solution of colloids such as albumin and polysaccharide will be used with the Hb-vesicle dispersion. The particle diameter is strictly adjusted to 250 nm. The degree of oxygen affinity is adjusted to a suitable value by coencapsulating an allosteric effector such as pyridoxal 5′-phosphate. The optimization of the composition of the lipid components resulted in high encapsulation efficiency of hemoglobin in the Hb-vesicle, a stability of 2 years in a liquid state, the prevention of hemolysis, an appropriate lifetime in blood circulation, and avoidance of platelet and complement activation. Furthermore, large-scale manufacturing has been improved by the introduction of freeze-thawing and freeze-drying operations which can control a molecular assembling state before encapsulating hemoglobin molecules.

Present Results of Safety and Efficacy Tests

Although in vivo testing was carried out using rats or hamsters, we confirmed the fundamental safety and oxygen transporting ability. Safety tests using primates is in progress. When 90% of the volume of rat blood was exchanged by the albumin dispersion of the Hb-vesicles, the oxygen partial pressure of the renal cortex was maintained as was blood pressure [4]. On the other hand, when the blood was exchanged by an albumin solution in the same concentration, the fall of blood pressure and oxygen partial pressure of the renal cortex became noticeable at 70% exchange, and all rats died just after 90% exchange.

In the hamster 80% exchange transfusion examination with the albumin dispersion of the Hb-vesicles, the noninvasively measured oxygen partial pressure of the subcutaneous tissue microcirculatory system was maintained at 5 times or more than that of the control albumin group although it fell to 60%–70% before exchange [5]. The contraction of a resistance blood vessel and the rise of blood pressure was not confirmed at all, but it was confirmed with modified hemoglobin products. Because the Hb-vesicle has a size that does not penetrate a blood vessel, there is no influence on the activity of nitrogen oxide as an endothelium-derived relaxation factor [6]. Furthermore, the Hb-vesicles cannot penetrate the sinusoidal vessels of liver (several holes 10–200 nm in size are open in the blood vessels) like old red blood cells, but are metabolized by Kupfer cells of liver and macrophages in reticuloendothelial systems. On the other hand, acellular hemoglobin molecules in the liver influenced liver microcirculation by eliminating carbon monoxide as a gaseous vasodilator, caused overgeneration of bilirubin, and suppressed bile secretion [7,8]. The half-life of Hb-vesicles in human blood circulation was estimated to be about three days on the basis of the results in rats, rabbits, and monkey. Moreover, from details of the blood biochemistry examination and pathology examination in the single and repetitive administration, we confirmed the transitional rise of lipase in connection with lipid decomposition, the transitional rise of a cho-

lesterol value, iron content, and the transitional hypertrophy of a reticuloendothelial system [9]. It was concluded that Hb-vesicles are expected to function adequately in cases of extensive bleeding.

Development of Platelet Substitutes

The history of platelet substitutes is short compared with that of red blood cells, with few examples of research. A platelet has complicated functions, such as adhesion specific to the bleeding site, expansion, aggregation, secretion, and the activation of a blood coagulation system. Needless to say, we cannot make platelet substitutes that have these all. However, a bleeding tendency is strongly apparent in such bleeding diseases as Bernard-Soulier syndrome and thrombasthenia, in which adhesion and aggregation ability are lacking. In these conditions, a hemostatic effect can be expected by the infusion of particles having functions such as adhesion and the aggregation of platelets due to the assistance of the function of the remaining platelets. Although clinical tests were carried out with human red blood cells [10] or albumin microcapsules [11] conjugating fibrinogen, and with the dried powder of human platelets [12], all clinical tests were suspended due to problems of safety and efficacy. Moreover, since the blood components of human origin were used, the risk of infection cannot be avoided completely. The platelet substitutes created by our group use liposomes and recombinant human albumin as biocompatible particles. They also use recombinant proteins of the part of platelet membrane or synthetic oligopeptides by conjugating to those particles for the purpose of accumulation to the bleeding site involving native platelets, expecting to achieve hemostasis.

The mechanisms of platelet adhesion differ between the blood flow of high shear rate and that of low shear rate. As shown in Fig. 2, the hemostasis of the platelets to bleeding in a high shear rate begins from a platelet recognition of the von Willebrand factor (vWf) bound to the collagen in the subcutaneous tissue of a blood vessel exposed to the bleeding site, followed by platelet adhesion and rolling. This recognition ability comes from the GPIb/V/IX complex containing GPIbα on the surface of a platelet [13]. Next, the platelets will progress and a granule will be secreted if GPIaIIa ($\alpha_2\beta_1$ integrin) or GPVI on the surface of the platelet directly interacts with the collagen and is activated. Fibrinogen recognizes the activated GPIIb/IIIa ($\alpha_{IIb}\beta_3$ integrin), constructs a crosslink between blood platelets, forms an aggregate, and serves as primary hemostasis. Hemostasis is completed by formation (secondary hemostasis) of the fibrin clot by induction of the coagulation system.

The target platelet substitutes bear the water-soluble part of receptor proteins such as GPIbα which recognizes collagen through vWf under the blood

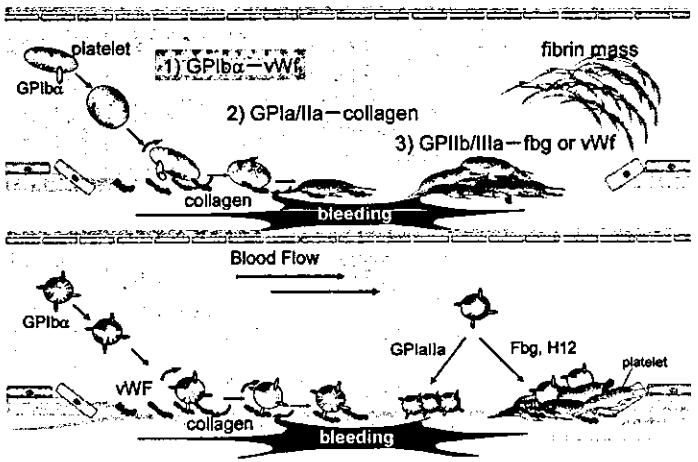

FIG. 2. Design of platelet substitutes studied from functions of natural platelets

flow of high shear rates and GPIaIIa which recognizes collagen directly at low shear rates. Instead of GPIIb/IIIa on the surface of an activated platelet, they bear the fibrinogen or its oligopeptide to assist the platelet aggregation as ligands of the GPIIb/IIIa.

If an anti-GPIIb/IIIa antibody is added to a platelet dispersion to inhibit GPIIb/IIIa activity, the platelets roll on the vWf-immobilized plate along the flow direction by the interaction of rGPIbα on the platelet and the vWf. Interestingly, phospholipid liposomes conjugating rGPIbα roll on the vWf-immobilized plate as well [14]. The number of the rolling liposomes increased with the shear rate, indicating the characteristic of rGPIbα. Moreover, the rolling speed was correlated with the membrane fluidity of the liposomes. That is, the rolling speed of "soft" liposomes was low, whereas that of the "hard" liposomes was high [15]. However, the rGPIbα-liposomes did not continue rolling but departed from the plate after rolling some length. This was remarkable as the "soft" liposomes. When the amount of rGPIbα on the surface of the liposome after the experiment was measured, it was suggested that rGPIbα-lipid should dissociate from the bilayer membrane during the rolling on the vWf-plate. Now rGPIbα-lipid which cannot dissociate serves as a point of a molecular design.

On the other hand, the rGPIaIIa-liposomes directly recognize collagen under the blood flow of low shear rates and adhered to (stopped at) the collagen-immobilized plate [16]. In this case, the number of the adhering liposomes decreased as the shear rate rose. However, liposomes conjugating both rGPIbα and GPIaIIa adhered on the collagen plate under the blood flow from low to high shear rates [17]. If the liposomes having platelet activation factors

or coagulation factors in the internal aqueous phase accumulate at the bleeding site, they will be able to contribute effectively to hemostasis by releasing their contents. We also focused on using polymerized albumin particles as effective platelet substitutes and obtained some unique in vitro and in vivo results [18,19].

Conclusions

For red-blood-cell substitutes the present target is the supportive treatment of transfusion therapy in emergency, and nonclinical and clinical studies will be scheduled within 2 years.

On the other hand, the research of platelet substitutes has just started. There is a conflict between the carrier design for the extension of circulation lifetime and the carrier design to show the hemostatic activity by recognizing the bleeding site; therefore, we need to resolve this conflict and to design platelet substitutes for prophylactic or chronic treatments. And we also need a method to confirm that the candidate does not create a thrombus in blood circulation.

Although profitability is important in the development of blood substitutes, one that is based on a long-term view with consideration for human beings is expected first. At present, as many discoveries about the dynamic function of platelets have accumulated in a short period of time with the progress of biotechnology and opto-electronics, and the manufacturing technology of recombinant proteins or carriers is progressing, a usable product is sure to be invented in the near future.

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Artificial Oxygen Carrier

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Safety and Efficacy of Hemoglobin-Vesicles and Albumin-Hemes

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Summary. Keio University and Waseda University have worked together on artificial O_2 carrier research for 20 years in close cooperation. Two candidate materials have been selected from the viewpoints of safety, efficacy, and cost performance. One is Hemoglobin-vesicles (HbV) and the other is albumin-heme (rHSA-heme). This chapter summarizes our video presentation that introduced the recent results of our research into HbV and rHSA-heme.

Key words. Blood substitutes, Oxygen carriers, Hemoglobin-vesicles, Albumin-heme, Oxygen therapeutics

Introduction: Keio-Waseda Joint Research Project

For human beings to survive, it is necessary to continuously deliver oxygen that is needed for the respiration of all tissue cells. Red blood cell, a so-called moving internal-organ, reversibly binds and releases O_2 under physiological conditions. From this point of view, red blood cell substitutes, or O_2-Infusions, are very important. In order to promote this research, we have emphasized that the establishment of basic science for macromolecular complexes and molecular assemblies is essential. We have systematically studied the Metal Complexes (synthetic heme derivatives) embedded into a hydrophobic cluster, and clarified that the electronic processes of the active sites are controlled by the surrounding molecular environment. Therefore, the reaction activity and its rate constant are observed as cooperative phenomena with the

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properties of the molecular atmosphere. In other words, the development of our O_2-Infusion has been based on "the Regulation of the Electronic Process on Macromolecular Complexes and Synthesis of Functional Materials" [1,2].

Reproducing the O_2-binding ability of red blood cells (RBC), that is, the development of a synthetic O_2 carrier that does not need hemoglobin (Hb), was the starting point of our study. In general, central ferric iron of a heme is immediately oxidized by O_2 in water, preventing the O_2 coordination process from being observed. Therefore, the electron transfer must be prevented. We were able to detect the formation of the O_2-adduct complex, but for only several nano seconds, by utilizing the molecular atmosphere and controlling the electron density in the iron center. Based on this finding, we succeeded in 1983 with reversible and stable O_2 coordination and preparation of phospholipid vesicles embedded amphiphilic-heme, known as lipidheme/phospholipids vesicles (Fig. 1) [3–6]. This was the world's first example of reversible O_2-binding taking place under physiological conditions. For example, human blood can dissolve about 27 ml of O_2 per dl, however a 10 mM lipidheme-phospholipid vesicle solution can dissolve 29 ml of O_2 per dl. This material is suitable for O_2-Infusion.

Soon after this discovery, Professor Kobayashi of Keio University asked Professor Tsuchida for a chance to evaluate the lipidheme solution with in vivo experiments. Since then the joint research and collaboration has continued since that time. We have synthesized over one hundred types of heme, and recently synthesized new lipidheme-bearing phospholipid groups, which complete self-organization in water to form stable vesicles. In 1985 Dr. Sekiguchi at Hokkaido Red Cross Blood Center proposed that Professor Tsuchida consider the utilization of outdated red blood cells and Hbs because, while the totally synthetic system is definitely promising it appeared that it

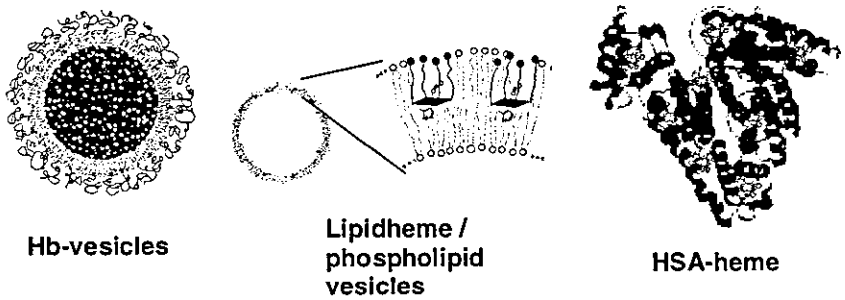


FIG. 1. Schematic representation of lipidheme-vesicle, hemoglobin-vesicle, and albumin-heme

would take considerable time to arrive at a social consensus for its use. We started to produce Hb-vesicles (HbV) using purified Hbs and molecular assembly technologies. In the late 1990's, a mass-production system for recombinant human serum albumin (rHSA) was established and we then prepared albumin-heme hybrids (rHSA-heme) using its non-specific binding ability, which is now considered to be a promising synthetic material.

Based on our effective integration of molecular science and technologies for functional materials developed by Waseda University, and the outstanding evaluation system of safety and efficacy developed by Keio University using animal experiments, we have made strong progress in our research on the O_2-Infusion Project. During this period, we have received substantial funding support from the Japanese government. In the near future, mass production and clinical tests of O_2-Infusion will be started by a certain pharmaceutical industry.

Background and the Significance of HbV

Historically, the first attempt of Hb-based O_2 carrier in this area was to simply use stroma-free Hb. However, several problems became apparent, including dissociation into dimers that have a short circulation time, renal toxicity, high oncotic pressure and high O_2 affinity. Since the 1970s, various approaches were developed to overcome these problems [7,8]. This includes intramolecular crosslinking, polymerization and polymer-conjugation. However, in some cases the significantly different structure in comparison with red blood cells resulted in side effects such as vasoconstriction [9].

Another idea is to encapsulate Hb with a lipid bilayer membrane to produce HbV that solves all the problems of molecular Hb [10]. Red blood cells have a biconcave structure with a diameter of about 8000 nm. Red blood cells can deform to a parachute-like configuration to pass through narrow capillaries. The possibility of infection and blood-type mismatching, and short shelf life are the main problems. Purified Hb does not contain blood-type antigen and pathogen, thus serves as a safe raw material for HbV.

HbV, with a diameter of 250 nm, do not have deformability but are small enough to penetrate capillaries or constrict vessels that RBC cannot penetrate. The surface of the vesicles is modified with polyethylene glycol (PEG) to ensure homogeneous dispersion when circulated in the blood and a shelf life of two years. The idea of Hb encapsulation with a polymer membrane mimicking the structure of RBC originated from Dr. Chang at McGill University [7]. After that, the encapsulation of Hb within a phospholipid vesicle was studied by Dr. Djordjevici at the University of Illinois in the 1970s [11]. However, it was not so easy to make HbV with a regulated diameter and ade-

quate O_2 transport capacity. We made a breakthrough in routinely producing HbV by using fundamental knowledge of macromolecular and supramolecular sciences. Some of the related technologies have already been published in academic journals [12–19]. Several liters of HbV are routinely prepared in a completely sterile condition. Hb is purified from outdated red blood cells, and concentrated to 40 g/dl. Virus removal is performed using a combination of pasteurization at 60°C and filtration with a virus removal filter. The Hb encapsulation with phospholipids bilayer membrane and size regulation was performed with an extrusion method. The vesicular surface is modified with PEG chains. The suspension of Hb-vesicles is dated at the final stage.

The particle diameter of HbV is regulated to about 250 nm, therefore, the bottle of HbV is turbid, and is a suspension. One vesicle contains about 30,000 Hb molecules, and it does not show oncotic pressure. There is no chemical modification of Hb. Table 1 summarizes the physicochemical characteristics of HbV. O_2 affinity is controllable with an appropriate amount of allosteric effectors, pyridoxal 5-phosphate. Hb concentration is regulated to 10 g/dl, and the weight ratio of Hb to total lipid approaches 2.0 by using an ultra pure and concentrated Hb solution of 40 g/dl, which is covered with a thin lipid bilayer membrane. The surface is modified with 0.3 mol% of PEG-lipid. Viscosity, osmolarity, and oncotic pressure are regulated according to the physiological conditions.

HbV can be stored for over two years in a liquid state at room temperature [17]. There is little change in turbidity, diameter, and P_{50}. MetHb content decreases due to the presence of reductant inside the HbV, which reduces the

TABLE 1. Physicochemical characteristics of HbV suspended in 5% albumin (HSA)

Parameters	HbV/HSA	Human blood (RBC)	Analytical method
diameter (nm)	220–280	8000	Light scattering method
P_{50} (Torr)	27–34[1]	26–28	Hemox Analyzer
[Hb] (g/dl)	10 ± 0.5	12–17	CyanometHb method
[Lipid] (g/dl)	5.3–5.9	1.8–2.5[2]	Molibuden-blue method
[Hb]/[Lipid] (g/g)	1.6–2.0	6.7[3]	—
[PEG-lipid] (mol%)	0.3	—	^{1}H-NMR
metHb (%)	<3	<0.5	CyanometHb method
viscosity (cP)[4]	3.7	3–4	Capillary rheometer
osmolarity (mOsm)	300	ca. 300	(suspended in saline)
oncotic press. (Torr)	20	20–25	Wescor colloid osmometer
pH at 37°C	7.4	7.2–7.4	pH meter
Endotoxin (EU/mL)	<0.1	—	LAL assay
Pyrogen	Free	—	rabbit pyrogen test

[1] Adjustable, [2] Total cell membrane components, [3] Weight ratio of Hb to total cell membrane components, [4] At 230 s^{-1}.

trace amount of metHb during storage. This excellent stability is obtained by deoxygenation and PEG-modification. Deoxygenation prevents metHb formation. The surface modification of HbV, with PEG chains prevents vesicular aggregation and leakage of Hb and other reagents inside the vesicles. Liquid state storage is convenient for emergency infusion compared to freeze-dried powder or the frozen state.

In Vivo Efficacy of HbV

The efficacy of HbV has been confirmed with isovolemic hemodilution and resuscitation from hemorrhagic shock. Some of the results have already been published in academic journals in the fields of emergency medicine and physiology [20–28]. In this chapter two important facts are described. One is isovolemic hemodilution with 90% blood exchange in a rat model. The other is resuscitation from hemorrhagic shock in a hamster model.

To confirm the O_2 transporting ability of HbV, extreme hemodilution was performed with HbV suspended in human serum albumin (HSA) [21,23] (Fig. 2). The final level of blood exchange reached 90%. Needle-type O_2 electrodes were inserted into the renal cortex and skeletal muscle, and the blood flow rate in the abdominal aorta was measured with the pulsed Doppler method. Hemodilution with albumin alone resulted in significant reductions in mean

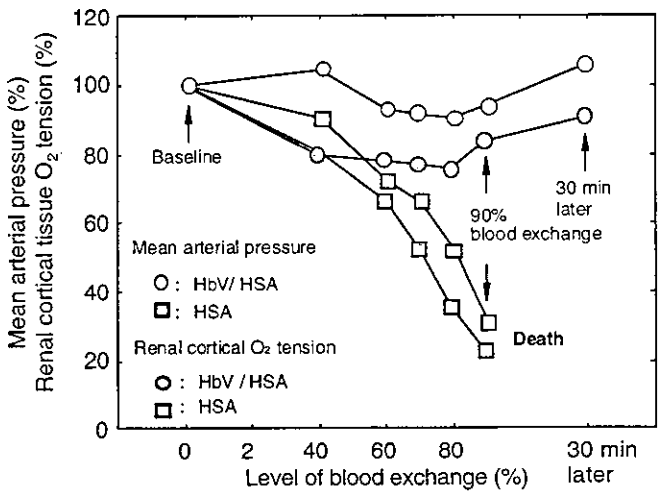


FIG. 2. 90% exchange-transfusion with HbV suspended in HSA (HbV/HSA), or HSA alone. Mean arterial pressure and renal cortical oxygen tension were monitored

arterial pressure and renal cortical O_2 tension, and finally all the rats died of anemia. On the other hand, hemodilution with HbV, suspended in HSA sustained both blood pressure and renal cortical O_2 tension, and all the rats survived. These results clearly demonstrate that HbV, has sufficient O_2 transporting capability.

To observe the microcirculatory response to the infusion of Hb products, we used the intravital microscopy equipped with all the units to measure blood flow rates, vascular diameter, O_2 tension, and so on. This system was developed by Professor Intaglietta at the University of California, San Diego. We used the hamster dorsal-skin fold preparation that allows observation of blood vessels from small arteries to capillaries. We evaluated the HbV suspension as a resuscitative fluid for hemorrhagic-shocked hamsters [26]. About 50% of the blood was withdrawn, and the blood pressure was maintained at around 40 mmHg for 1 h. The hamsters either received HbV suspended in HSA (HbV/HSA), HSA alone, or shed blood (Fig. 3). Immediately after infusion, all the groups showed increases in mean arterial pressure, however, only the albumin infusion resulted in incomplete recovery. The HbV/HSA group showed the same recovery with the shed autologous blood infusion. During the shock period, all the groups showed significant hyperventilation that was evident from the significant increase in arterial O_2 partial. Simultaneously,

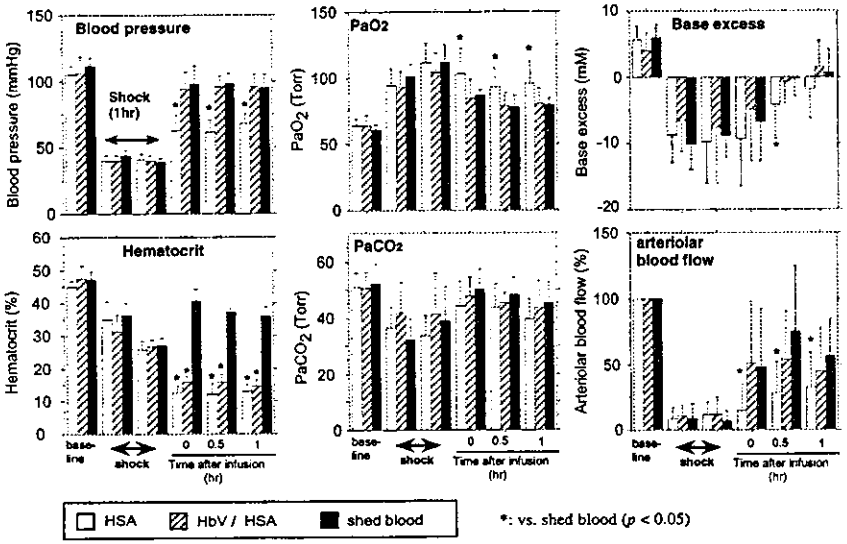


FIG. 3. Resuscitation from hemorrhagic shock with HbV suspended in HSA (HbV/HSA) in hamster dorsal skinfold model. Mean ± SD

base excess and pH decreased significantly. Immediately after resuscitation, all the groups tended to recover. However, only the HSA group showed sustained hyperventilation. Base excess for the HSA group remained at a significantly lower value one hour after resuscitation. Blood flow decreased significantly in arterioles to 11% of basal value during shock. The HbV/HSA and shed autologous blood groups immediately showed significant increases in blood flow rate after resuscitation, while the albumin group showed the lowest recovery.

In Vivo Safety of HbV

We further examined the safety profile of HbV such as cardiovascular responses, pharmacokinetics, influence on reticulo endothelial system (RES), influence on clinical measurements and daily repeated infusion [29–35].

We observed the responses to the infusion of intra-molecularly cross-linked Hb (XLHb) and HbV into conscious hamsters. XLHb (7 nm in diameter) showed a significant increase in hypertension equal to 35 mmHg, and simultaneous vasoconstriction of the resistance artery equal to 75% of the baseline levels [30] (Fig. 4). On the other hand, HbV at 250 nm, showed minimal change. The small acellular XLHb is homogeneously dispersed in the plasma, and it diffuses through the endothelium layer of the vascular wall and reaches the smooth muscle. Intra-moleculary cross-linked Hb traps nitric oxide (NO) as an endothelium-derived relaxation factor, and induces vasoconstriction, and hypertension. On the other hand, the large HbV stay in the lumen and do not induce vasoconstriction. Several mechanisms are proposed for Hb-induced vasoconstriction. These include NO-binding, excess O_2 supply, reduced shear stress, or the presence of Hb recognition site on the

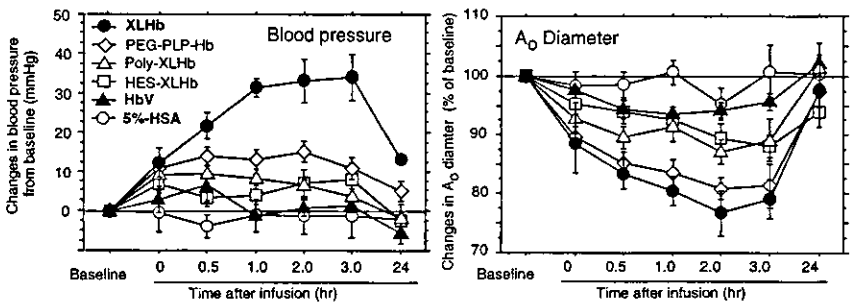


FIG. 4. Changes in mean arterial pressure and the diameters of the resistance artery in hamster dorsal skin microcirculation after the bolus infusion of Hb-based O_2 carriers. Mean ± SD

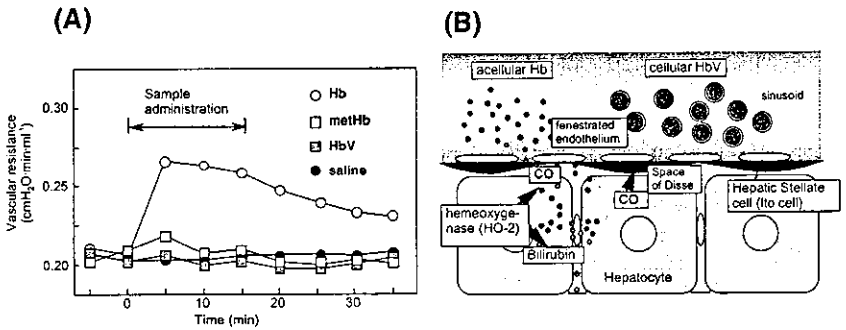


FIG. 5. A Changes in vascular resistance during perfusion of exteriorized rat liver with HbV, Hb, metHb, or saline. B Schematic representation of hepatic microcirculation: The small Hb molecule extravasate across the fenestrated endothelium to reach to the space of Disse, where heme of Hb is catabolised by hemeoxygenase-2 (HO-2) and CO is released as a vasorelaxation factor. However, the excess amount of the extravasated Hb traps CO and induces vasoconstriction and the resulting higher vascular resistance. On the other hand, the larger HbV retains in the sinusoid and there is no extravasation and vasoconstriction

endothelium. But it is clear that Hb-encapsulation shields against the side effects of acellular Hbs.

Professor Suematsu at Keio University has revealed the effects of Hb-based O_2 carriers in hepatic microcirculation [29,32] (Fig. 5). On the vascular wall of the sinusoid in hepatic microcirculation, there are many pores, called fenestration, with a diameter of about 100 nm. The small Hb molecules with a diameter of only 7 nm extravasate through the fenestrated endothelium and reach the space of Disse. On the other hand, HbV particles, which are larger than the pores, do not extravasate. Heme of extravasated Hb is excessively metabolized by hemeoxygenase-2 in hepatocyte to produce CO and bilirubin. Even though CO acts as a vasorelaxation factor in the liver, the excess amount of Hb rapidly binds CO, resulting in the vasoconstriction and an increase in vascular resistance. On the other hand, HbV (250 nm in diameter) is large enough to remain in the sinusoid, and the vascular resistance is maintained.

So, what is the optimal molecular dimension of Hb-based O_2 carriers? The upper limitation is below the capillary diameter to prevent capillary plugging, and for sterilization by membrane filters (Fig. 6). On the other hand, smaller sizes exhibit a higher rate of vascular wall permeability with side effects such as hypertension and neurological disturbances. HbV exhibits a very low level of vascular wall permeability. Therefore, the HbV appears to be appropriate from the viewpoint of hemodynamics. We have clarified the influence of HbV on the RES, because the fate of HbV is RES trapping.

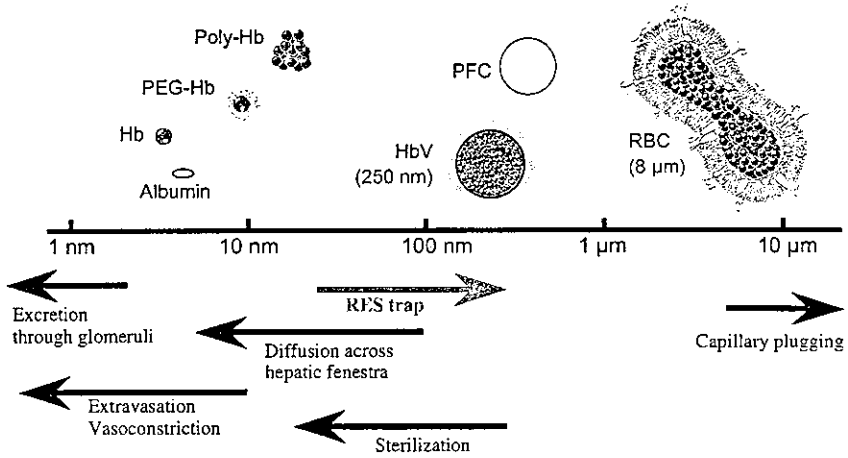
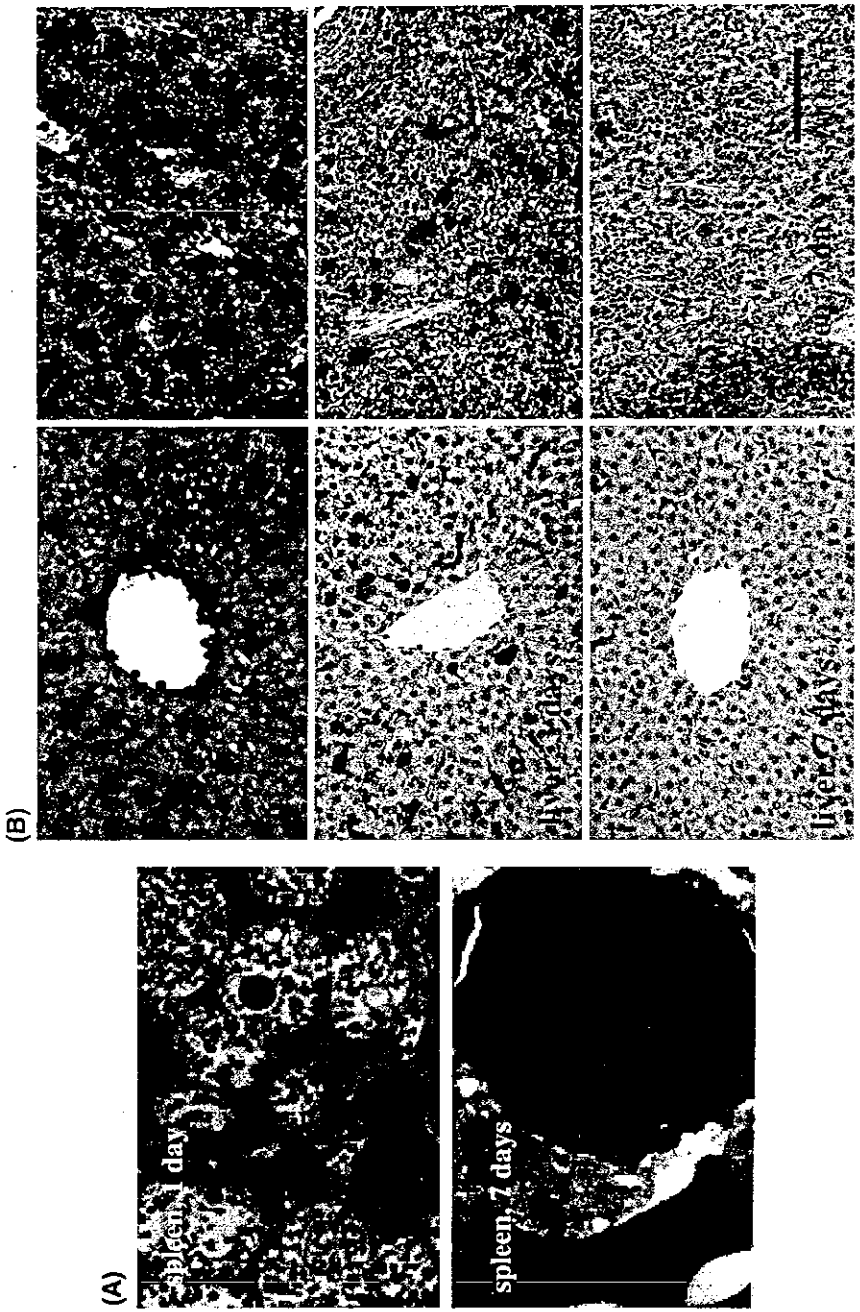


FIG. 6. Optimal diameter of Hb-based oxygen carriers from the view point of physiological response and production process

Circulation persistence was measured by monitoring the concentration of radioisotope-labeled HbV in collaboration with Dr. W.T. Phillips at the University of Texas at San Antonio. The circulation half-life is dose dependent, and when the dose rate was 14 ml/kg, the circulation half-life was 24 h. The circulation time in the case of the human body can be estimated to be twice or three times longer; or about 2 or 3 days at the same dose rate. Gamma camera images of radioisotope-labeled HbV showed the time course of biodistribution. Just after infusion, HbV remains in the blood stream so that the heart and liver that contain a lot of blood showed strong intensity. However, after it is finished playing its role in O_2 transport, a total of 35% of HbV are finally distributed mainly in the liver, spleen and bone marrow.

The time course of liver uptake was monitored with a confocal fluorescence microscope. Hb-vesicle was stained with a lipid fluorophore. The liver of an anesthetized hamster was exposed and a fluorescence-labeled-HbV was infused intravenously. Due to the motion of respiration, the picture oscillates. However, a static frame can be obtained. The individual particles of HbV cannot be recognized. When the vesicles are accumulated in phagosomes of Kupffer cells, they can be recognized with a strong fluorescence. How is HbV metabolized in macrophages? The transmission electron microscopy (TEM) of the spleen 1 day after infusion of HbV clearly demonstrated the presence of HbV particles in macrophages, where HbV particles that appear as black dots are captured by the phagosomes [34] (Fig. 7). Red blood cells and HbV contain a lot of ferric iron with a high electron density, so that they show



strong contrast in TEM. However, after 7 days, the HbV structure cannot be observed. We confirmed no abnormalities in the tissues and no irreversible damage to the organs or complete metabolism within a week. A Polyclonal anti-human Hb antibody was used as the marker of Hb in the HbV. This antibody does not recognize rat Hb. The red-colored parts indicate the presence of Hb in HbV, and almost disappear after 7 days in both the spleen and liver. This shows that HbV can be metabolized quite promptly.

One issue of the Hb-based O_2 carriers is that they have a significant influence on clinical laboratory tests. They remain in the plasma phase in hematocrit capillaries after centrifugation of blood samples, and interfere with the colorimetric and turbidimetric measurements. However, HbV can be simply removed from blood plasma either by ultracentrifugation or centrifugation in the presence of a high-molecular-weight dextran to enhance precipitation. We can obtain a very clear supernatant for accurate analyses [35]. This is one advantage of HbV in comparison with acellular Hb solutions. Accordingly we examined the influence on organ functions by serum clinical laboratory tests after the bolus infusion of HbV at a dose rate of 20 ml/kg. Albumin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and lactic dehydrogenase (LDH), which reflect the liver function, moves their values within normal range. Concentrations of bilirubin and ferric ion are maintained at a low level. The concentration of lipids transiently changed. In particular, the cholesterols increased significantly. And phospholipids slightly increased, however, they returned to the original level after 7 days. These results indicate that the membrane components of HbV, once they reappear from RES, are metabolized on the physiological pathway.

We recently tested a daily repeated infusion of HbV in Wistar rats as a safety study. The dose rate was a 10-ml/kg/day infusion for 14 days. All rats well tolerated and survived. Body weight showed a monotonous but slightly depressed increase in comparison with the saline group. However, after 2 weeks there was no significant difference with the saline control group. All the rats seemed very healthy and active. There was no piloerection. As for the hematological parameters, the numbers of white blood cells and platelets did not exhibit a significant difference from the HbV group and the saline control group. Hematocrit showed a slight reduction for the HbV group, probably due to the accumulation of the large amount of HbV in the blood. Histopathological examination one day after the final infusion of HbV showed signifi-

FIG. 7. A Transmission electron microscopy of rat spleen one day after the infusion of HbV (20 ml/kg) and after 7 days. Black dots are HbV particles captured in phagosomes in the spleen macrophages, and they disappeared at 7 days. B Staining with anti-human Hb antibody showed the presence of HbV in spleen and liver. They disappeared within 7 days. Cited from: Sakai et al (2001) Am J Pathol 159:1079–1088

cant accumulation of HbV in spleen macrophages, and liver Kupffer cells. Berlin Blue staining revealed the presence of hemosiderin indicating that the metabolism of Hb was initiated. There were no other morphological abnormalities, and the serum clinical chemistry indicated transient but reversible increases in lipid components. AST and ALT were within the normal range. From these results we are confident with the safety of HbV.

Design and Physicochemical Properties of rHSA-Heme

We have been conducting research on totally synthetic O_2 carriers, or so-called albumin-heme that does not require Hb. Human serum albumin is the most abundant plasma protein in our blood stream, but its crystal structure has not been elucidated for long time. In 1998, Dr. Stephen Curry of the Imperial College of London was the first elucidate the crystal structure of the human serum albumin complexed with seven molecules of myristic acids [36]. He found that the dynamic conformational changes of albumin take place by the binding of fatty acid.

In Japan, recombinant human serum albumin is now manufactured on a large scale by expression in the yeast Pichia pastoris, and it will appear on the market soon [37]. A large-scale plant, which can produce one million vials per year, has been already established. From the viewpoint of clinical application, O_2-carrying albumin is quite exciting and may be of extreme medical importance. With this background, we have found that synthetic heme derivative is efficiently incorporated into recombinant human serum albumin (rHSA), creating a red-colored rHSA-heme hybrid. This rHSA-heme can reversibly bind and release O_2 molecules under physiological conditions in the same manner as Hb. In other words, our rHSA-heme hybrid is a synthetic O_2-carrying hemoprotein, and we believe that its saline solution will become a new class of red blood cell substitute. We have already published these chemistry findings and technologies in international journals [38–49].

Figure 8 summarizes the structure of the albumin-heme molecule. The Maximal binding numbers of heme to one albumin are eight, and the magnitude of the binding constants ranged from 10^6 to 10^4 (M^{-1}). The isoelectric point of rHSA-heme was found to be 4.8, independent of the binding numbers of heme. This value is exactly the same as that of albumin itself. Furthermore, the viscosity and density did not change after the incorporation of heme molecules, and the obtained solution showed a long shelf life of almost two years at room temperature. The O_2-binding sites of rHSA-heme are iron-porphyrin, therefore the color of the solution changed in a similar way to Hb. Upon addition of O_2 gas through this solution, the visible absorption pattern immediately changed to that of the O_2-adduct complex. Moreover, after bubbling carbon monoxide gas, albumin-heme formed a very stable carbonyl complex.

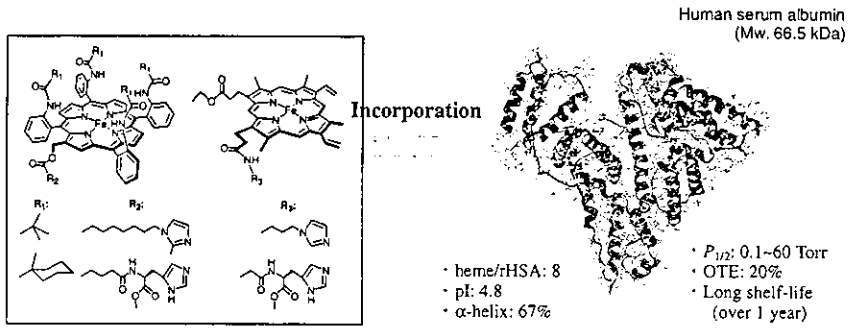


FIG. 8. Structure of the albumin-heme molecule

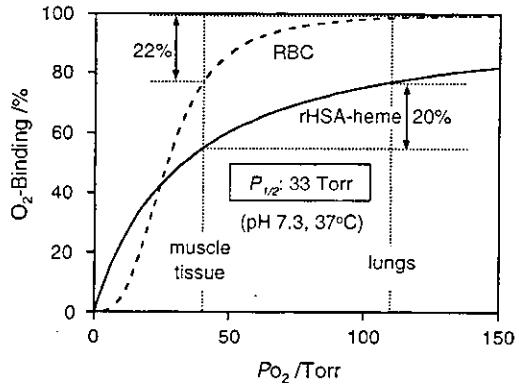


FIG. 9. O_2-binding equilibrium curve of albumin-heme

Figure 9 shows the O_2-binding equilibrium curve of rHSA-heme. The O_2-binding affinity, of rHSA-heme is always constant, independent of the number of heme, and the O_2-binding profile does not show cooperativity. However, the O_2-transporting efficiency of albumin-heme between the lungs where PaO_2 is 110 Torr and muscle tissue where PtO_2 is 40 Torr increases to 20%, which is similar to 22% efficiency of red blood cells. The O_2-binding property of albumin-heme can be controlled by changing the chemical structure of heme derivatives incorporated. More recently, we have found that a protoheme derivative is also incorporated into albumin and can bind and release O_2 as well [50].

In Vivo Safety and Efficacy of rHSA-Heme

Based on these findings, we can say that rHSA-heme can become an entirely synthetic O_2-carrier, and satisfy the initial clinical requirements for a red blood cell substitute. However, we have another problem to solve before we can use this material as an O_2-carrier in the circulatory system. This problem is NO scavenging. Of course, it can bind NO, and it may be anticipated that the injection of rHSA-heme also induce hypertensive action. We have evaluated the efficacy and safety of this rHSA-heme solution with animal experiments.

As described before, small Hb molecules extravasate through the vascular endothelium and react with NO, thus inducing vasoconstriction and acute increases in systemic blood pressure. Contrary to the expectations, the observation of the intestinal microcirculation after the infusion of rHSA-heme into an anesthetized rat revealed that the diameters of the venules and arterioles were not deformed at all [51]. Indeed, only a small change in the mean arterial pressure was observed after the administration of the rHSA-heme solution (Fig. 10). In contrast, the infusion of Hb elicited an acute increase in blood pressure. Why does albumin-heme not induce vasoconstriction or hypertension? The answer probably lies in the negatively charged molecular surface of albumin. One of the unique characteristics of serum albumin is its low permeability through the capillary 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.

We are now evaluating the O_2-transporting ability of this albumin-heme molecule in the circulatory system with further animal experiments [52]. First, we determined the physiological responses to exchange transfusion with rHSA-heme solution into rats after 70% hemodilution and 40% hemorrhage

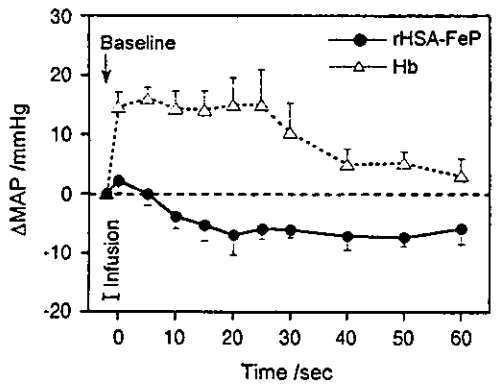


FIG. 10. Change of MAP after the administration of rHSA-heme solution in the anesthetized rats (n = 5). All data are shown as changes from the basal values (ΔMAP) just before the infusion and expressed as mean ± S.E. Basal value is 90.1 ± 3.0 mmHg

(Fig. 11). The declined mean arterial pressure and blood flow after a 70% exchange with albumin and further 40% bleeding of blood showed a significant recovery of up to 90% of the baseline values by the infusion of the rHSA-heme solution. On the other hand, all rats in the control group only injected with albumin died within 30 min. Furthermore, muscle tissue O_2-tension significantly increased. These responses indicate the in vivo O_2-delivery of the rHSA-heme solution.

More recently, we have synthesized human serum albumin dimer, which can incorporate sixteen hemes in its hydrophobic domain [53]. The human serum rHSA-heme dimer solution dissolves 1.2 times more O_2 compared to that of red blood cells and keeps its colloid osmotic pressure at the same level as the physiological value.

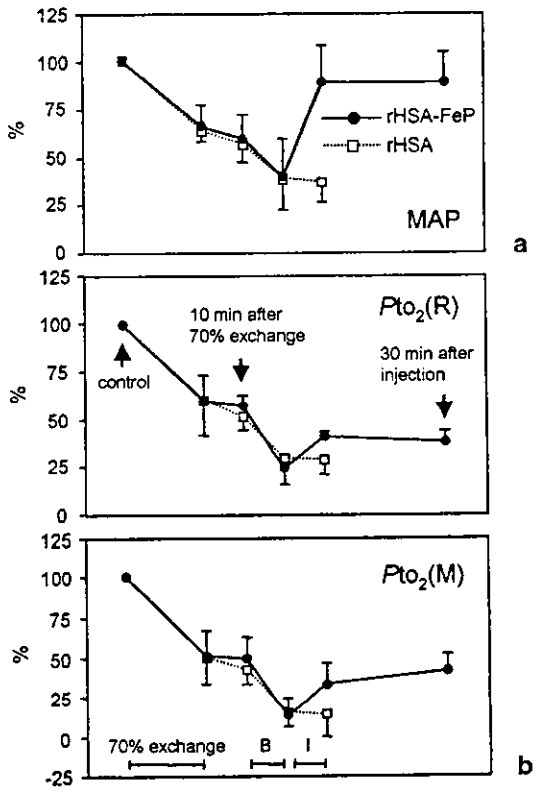


FIG. 11. Change of (a) MAP and (b) O_2-tension in renal cortex during the 70% hemodilution with 5 wt% rHSA and further 40% exchange transfusion with rHSA-heme in anesthetized rats (n = 5). All data are shown as changes from the basal values and expressed as mean ± S.E.