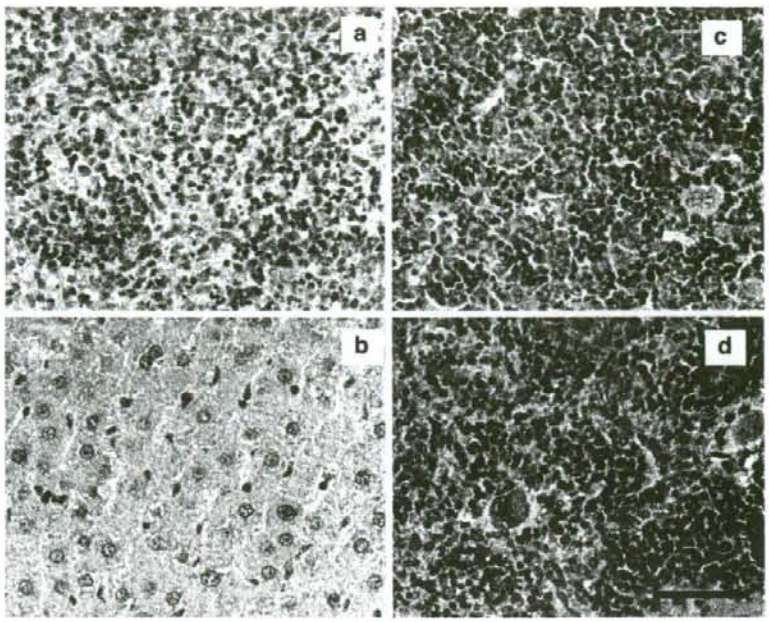

図5 HbV/rHSA 群および sRBC/rHSA 群の 14 日後の組織切片のベルリンブルー染色像. HbV/rHSA 群の (a) 脾臓, (b) 肝臓, および (c) 骨髄. 脾臓と肝臓にヘモジデリンが存在した. (d) sRBC/rHSA 群の脾臓にもヘモジデリンが検出された. スケールバーは 50μm. (From : Sakai et al., Transfusion 2006 ; 46 : 339-347, Blackwell Publishing, Oxford, UK)

が次第に減少し, 14 日後には消失した. HbV/rHSA 群では 3 日後に血中リバーゼの上昇が認められたが, 膵臓に著変は認められなかった. HbV/rHSA 群の脾臓のギムザ染色では, 1, 3 日後に赤脾髄に捕捉された HbV が多く認められた. しかし 7 日後には減少し, 14 日後には消失していた (図 4). 実験期間を通して脾索に赤芽球および前赤芽球の巣 (集合) が多く存在し, 特に 3, 7 日後で顕著であった. 巣の形成から, 活発な髄外造血が伺えた. rHSA 群でも 3 日目に赤芽球巣を多く認めた. 骨髄での造血も HbV/rHSA 群で確認された. ベルリンブルー染色により, HbV/rHSA 群で 7 日後に脾臓にヘモジデリンが僅かに観測され, 14 日後に更に顕著になった (図 5). 僅かに肝臓のクッパー細胞にも認められたが, 骨髄には無かった. sRBC/rHSA 群では, 14 日後に脾臓にヘモジデリン沈着が観測された.

4. 考　察

本研究における重要な点は, HbV/rHSA 溶液により 40% 血液交換されて低下した Hct 値が, 7 日後には完全に回復したこと, また, 14 日までに RES に捕捉された HbV がほぼ消失したことである. HbV 群の脾臓肥大は, 赤脾髄への HbV の捕捉と, EPO 分泌に反応して強い造血作用により赤芽球巣が脾索に多く存在する事に起因すると考えられた. しかし脾臓重量は 14 日後には完全に回復していた.

これまでの放射化ラベルした HbV の体内動態の研究から, HbV は最終的に RES に移行することが解っている[1)16)]. 脾臓肥大の一因は, 図 4 から解るように, HbV が赤脾髄に捕捉されることであるが, 14 日後には消失する. 投与 3 日後までの血中コレステロールの緩やかな上昇, また HbV が血中では分解しない事実から, HbV が RES のマクロファージの食胞で捕捉分解されてからコレステロールが血中に遊離したものと考えられた[14)15)]. 既報の HbV 負荷投与試験の結果では, 高密度リポ蛋白コレステロール, β リポ蛋白, リン脂質の増大が, 過剰量として血中に確認された[15)17)]. しかし, 今回の 40% 交換輸血試験では, それらの上昇が見られず, むしろ低下する傾向もあった. 従って大量の血液が HbV/rHSA で置換された場合には, HbV の脂質成分が体内で有効利用され, 造血や成長に使用された可能性がある.

HbV が大量に投与され, その分解過程でヘム鉄やポルフィリンの分解物であるビリルビンが大量に遊離することを懸念したが, 血中濃度の上昇は 14 日間全く見られなかった. Hb から放出されたヘムは, 肝臓クッパー細胞や, 脾臓マクロファージの誘導型ヘムオキシゲナーゼ-1 の酵素分解を受け, ビリルビンに変化する[15)32)]. 今回の実験から, HbV の大量投与に際してもビリルビンは通常の経路によって分解され, 胆汁となって排泄さ

れること，また胆汁管排泄機能に影響が無いことが考えられた．ベルリンブルー染色では，14日後の肝臓と脾臓にヘモジデリン沈着が認められた．通常，ヘム由来の鉄はフェリチンとして蓄えられるが[33]，鉄含量が多くなるとヘモジデリンになる．フェリチンもヘモジデリンも鉄イオンを遊離し，過酸化水素とのフェントン反応によりOHラジカルを産生し，これが脂質過酸化を助長する懸念がある[34]．しかし化学的には，不溶化沈着したヘモジデリンの方がフェリチンよりも不活性であるとの見方もある[35]．一般的に輸血を頻回受ける患者では，保存赤血球の血中半減期が短いためRESに捕捉され，ヘモジデリンが多く認められる．本研究では，sRBC群でも緩慢ではあるが脾臓肥大とヘモジデリン沈着が認められた．保存血では老化赤血球のように赤血球の変形能が低下し脆弱となり，血中滞留時間が短くなり脾臓で捕捉，分解されることも原因の一つと考えられる[26]．これらのことから，HbV由来のヘムの分解および鉄の貯蔵は，生理的許容範囲内であり，脆弱な保存赤血球の場合と同様の良く知られた生理的経路により行われていることが予想できた[36]．

興味深い事に，脾臓肥大はHbV/rHSA群およびsRBC/rHSA群だけでなく，rHSA群で3日目に顕著であった．文献によると，ラットは低酸素状態に曝されると，髄外造血が脾臓で顕著になる[37][38]．我々の実験では，rHSA群で特に3日目に大量の赤芽球巣の存在を見出している．^{125}I-rHSAの体内動態に関する文献から[39][40]，rHSAが異種蛋白質として脾臓に特異的に捕捉される可能性は低く，脾臓肥大との関係も無いと考えられる．従ってrHSA群の脾臓肥大は，EPO分泌にによって髄外造血が活性化された為と考えられた．

腎臓から分泌されるEPOは，貧血の状態を強く反映する[41][42]．rHSA群で投与1日後に最も高いEPO値を示し，酸素運搬量の低下により貧血状態が最も強かったことが伺える．次いでHbV/rHSA群が高値を示した．これはHbVの血中半減期が短い事と，酸素運搬機能の無いmetHbの含量が増大することに起因している[43]．また，sRBC/rHSA群でも血液交換後にHctが低下しているため，EPO値の緩慢な上昇が確認された．従って，HbV/rHSA群およびsRBC/rHSA群の脾臓肥大のもう一つの要因として，造血過程における赤芽球巣の存在があり，これがHctを1週間で回復させた原動力であると考えられる．興味深い事に，rHSA群，HbV/rHSA群の14日後のHct値は46%となり，sRBC群の値(43%)を上回っていた．これはEPO分泌量がrHSA群，HbV群がsRBC群よりも高く，造血が亢進されたためovershootingしたのかもしれない．MCH，MCV，MCHCが正常であったことも，造血作用に異常が無いことを支持している．

血液生化学検査では，HbVが大量に肝臓クッパー細胞に移行するにも関らず，特に肝機能に影響が無いことを示唆した．HbVの投与によってリパーゼの上昇が既報の負荷投与試験も含め常に認められている．これは，HbVの成分である脂質によって，リパーゼの分泌が亢進したのかもしれない[15][17][44]．

結論として，臨床でのHbVの使用を想定したラットへの投与試験（40% 血液交換）に際し，ラットは全例が生存し，血液希釈により低下したHctは7日以内に回復した．一過性の脾臓肥大とヘモジデリン沈着が確認されたが，余剰な鉄イオンの遊離は認められなかった．HbVは脆弱な保存赤血球の場合と同様の生理的経路により，細網内皮系で捕捉代謝され，構成成分が支障無く排泄或は再利用される過程が予想された．まだ不明の点もあるが，HbVを輸血代替として使用する場合の安全性についての知見を得ることができた．

謝辞：試料調製を担当した宗慶太郎助教授，政田洋平氏(早稲田大学)，組織病理学的検討に際し染色を担当した阿部仁氏（慶應義塾大学医学部病理学教室），また有益なご助言を頂きました末松誠教授（慶應義塾大学医学部医化学），ならびに村田満教授（慶應義塾大学医学部臨床検査医学）に感謝申し上げます．リコンビナントアルブミンはニプロ社より提供を受けた．本研究の一部は，厚生労働科学研究費補助金(医薬品・臨床機器等レギュラトリーサイエンス総合研究事業)，日本学術振興会 科学研究費補助金(B16300162)，日本人工臓器学会JSAO-Grantにより推進された．また，(株) オキシジェニクスの支援を受けた．記して謝意を表する．

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ACUTE 40 PERCENT EXCHANGE-TRANSFUSION WITH HEMOGLOBIN-VESICLES (HBV) SUSPENDED IN RECOMBINANT HUMAN SERUM ALBUMIN SOLUTION: DEGRADATION OF HBV AND ERYTHROPOIESIS IN A RAT SPLEEN FOR 2 WEEKS

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Keywords:
Artificial Blood, Artificial Oxygen Carriers, Artificial Red Cells, Liposome, Plasma Substitutes

Journal Web Site: http://yuketsu.gr.jp

トピックス

ヘモグロビン小胞体を用いた人工心肺充填液による高次脳機能保護効果 －ラット人工心肺モデルによる検討－

Use of Hemoglobin Vesicles during Cardiopulmonary Bypass Priming Prevents Neurocognitive Decline in Rats

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研究要旨

新生児や乳児の開心術の成績は近年飛躍的に向上しているが，一般的には人工心肺回路の充填液として輸血が必須である．これは低体重の患者において無輸血充填を行った場合，高度の血液希釈が生じ，特に酸素需要の大きな脳の不可逆的障害を来たす可能性が高いためである．一方で，輸血には感染症，移植片対宿主反応，免疫抑制，炎症性生体物質活性化による臓器障害といった合併症の危険を伴う．こうした臨床上のジレンマの一解決手段として，我々は早稲田大学理工学総合研究センター及び慶應義塾大学呼吸器外科にて共同研究が進められているヘモグロビン小胞体（Hemoglobin vesicle, HbV）に着目した．本研究は，ラット人工心肺モデルを確立した後，人工心肺回路をHbVで充填した群において高次脳機能が維持されることを証明した．これはHbVの充填により末梢組織への酸素運搬が保持されたためと考えられ，HbVの有用性が明らかとなった．

A　緒言

先天性心疾患を伴う体重10kg以下の乳児の開心術においては，同種血輸血による人工心肺回路充填が一般に行われている．その理由は，人工心肺回路の充填液は300mlから400ml必要であり，これを晶質液で満たした場合，体重10kg以下の患者（循環血液量は約800ml）では，高度な血液希釈が生じ，酸素運搬を担う赤血球の相対的な減少により組織障害，特に酸素需要の大きな脳の障害を来たすためである．

このために，現在体重10kg以下の患者に体外循環を行う場合，赤血球輸血は避けられない状況にある．しかし一方で，輸血による感染症，移植片対宿主反応，免疫抑制といった合併症のリスクを伴い，社会的にも大きな問題となっている．また，炎症性生体物質の遊離を促進することによる脳障害の発生も指摘されており，できるだけ輸血を避けるように努めるべきである．

この臨床上のジレンマの解決手段として，我々は，人工酸素運搬体であるHbVに着目した[1-3]．現時点のHbVは半減期が約20から30時間と短い．しかしながら人工心肺運転中に生じる血液希釈状態という特殊な環境はおよそ数時間であり，その数時間だけ血液の役割を果たし，その後速やかに代謝される現在のHbVは小児心臓外科の立場から考えると臨床上のジレンマを解決に導く大きな利点となる．すなわち，HbVの短い半減期を活用するという逆転的発想により本研究は成り立っている．

本研究は，ラット人工心肺モデルにおいてHbVを用いた人工心肺充填液による高次脳機能保護効果の検討を目的に行われた．

B　方法

人工酸素運搬体は早稲田大学理工学総合研究センター及び慶應義塾大学呼吸器外科にて共同研究が進められているHbVを使用した[4]．ローラーポンプと特製膜型肺を用いて人工心肺回路を作成し（Fig. 1.）[5]，回路内に5%アルブミンを充填した群（HbV (-) prime群　n=7），HbVを充填した群（HbV (+) prime群 n=7），偽手術群（sham surgery群 n=7）の3群に分けて実験を行った．体重450g前後のSDラットをセボフルレンにて全身麻酔し，14G静脈留置針にて気管内挿管した．挿管後人工呼吸器管理とした．人工心肺の確立に際して，脱血管は右内頸静脈を介して右房へ，送血管は尾動脈に挿入した．胸骨正中切開は行わなかった．右内頸静脈にアプローチする際，ラットの右頸部に切開を加えるが，皮切線はわずかで動物への負担を最小にするように配慮した．人工心肺の運転は常温下，無拍動送血法で200ml/kg/minの流量で90分間行った．回路内充填量は60mlとした．HbV充填液のヘモグロビン濃度は8.6g/mLとした（Fig. 2.）．

人工心肺運転終了後は人工心肺回路内の残存血液を遠心分離し，沈殿した自己血を20分間かけて血管内に戻した．その後，

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論文受付 2006年5月29日　論文受理 2006年7月14日

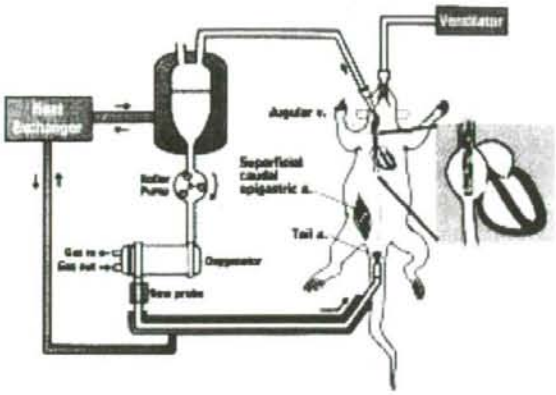

Fig. 1. Schema of cardiopulmonary bypass in a rat.

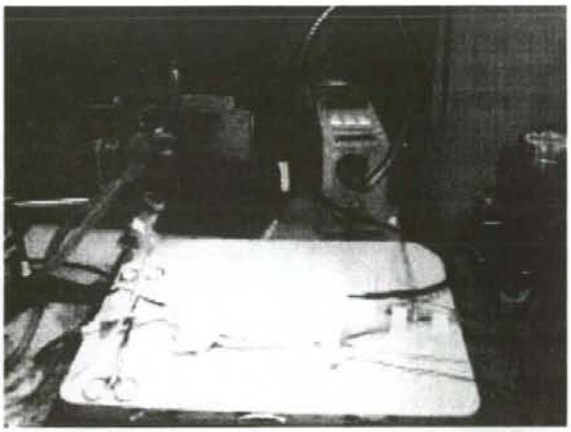

Fig. 2. Establishment of cardiopulmonary bypass in a rat.

脱血管及び送血管を抜去し，麻酔から覚醒させた後，ゲージにて飼育した．術後1，3，5，7日目に標準化神経学的機能試験及び2種の迷路試験（Morris water maze test）を行った[6,7]．標準化神経学的機能試験はneurologic performance scale, Functional disability scoresを用いて数値化した（Neurologic performance scale : 0-95, 0 = no deficit and 95 = brain death, Functional disability scores ：score 1 = no disability; score 2 = mild disability; score 3 = moderate disability; score 4 = severe disability; and score 5 = death.）[8]．

以上の試験はビデオ録画して，後に盲検となっている神経学者が一括して評価した．術後7日目に動物は犠牲死させ，脳の組織を採取し，海馬部の病理学検査を行った．

C. 結果

1．人工心肺運転中の検査所見

HbV (-) prime，HbV (+) prime，sham surgeryの3群における人工心肺運転中の採血データを検討した結果，Arterial pHに関してはHbV (-) prime群でアシドーシスの傾向があった．これは血液希釈によるものと考えられた．またHbVの充填に関わらず，人工心肺を運転した群に関しては人工心肺運転後においてArterial PCO_2の上昇を認めた．ただし，それ以外の大きな所見は得られなかった．3群ともいずれの症例も生存した．

2．標準化神経学的機能試験および迷路試験

術後1，3，5，7日目に標準化神経学的機能試験及び2種の迷路試験（Morris water maze test）を行った．各群のswimming speedには有意差がなかった．標準化神経学的機能試験はNeurologic performance scaleおよび，Functional disability scoreを用いておこなったが，3群ともに有意差を認めなかった．

迷路試験は認知記憶の評価で行われるもので，高次脳機能に関わる海馬の評価に用いられる．water maze test-1はFig. 3.に示すとおり，円形のプールに浮島を設けたもので一般的にMorris water maze testと呼ばれるものである．この結果からHbV (-) prime群は，HbV (+) prime群及びsham surgery群と比較して有意に到着時間が延長したことが分かった（p=0.005）．またHbV (+) prime群とsham surgery群には有意差がなかった．water maze test-2はFig. 4.に示すとおり，実際に迷路を正方形の枠の中に作成したものである．11箇所のjunction pointを設けた．この結果からもHbV (-) prime群は，HbV (+) prime群及びsham surgery群と比較して有意に到着時間が延長したことが分かった（p=0.05）．またHbV (+) prime群とsham surgery群には有意差がなかった．

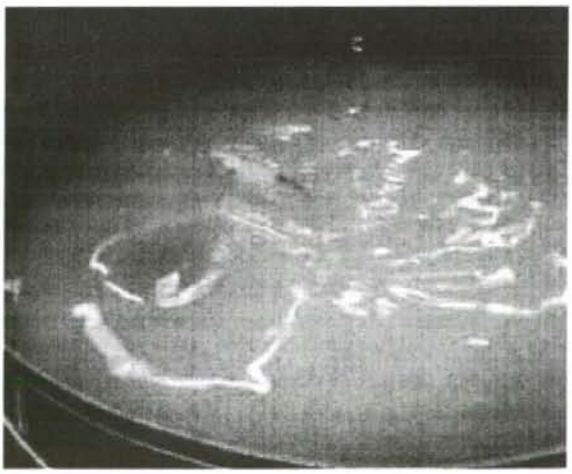

Fig. 3. Water maze test - 1
Neurocognitive outcome was assessed on the 1st, 3rd, 5th, and 7th days after CPB by visual-spatial learning with the maze test (Water maze test - 1).

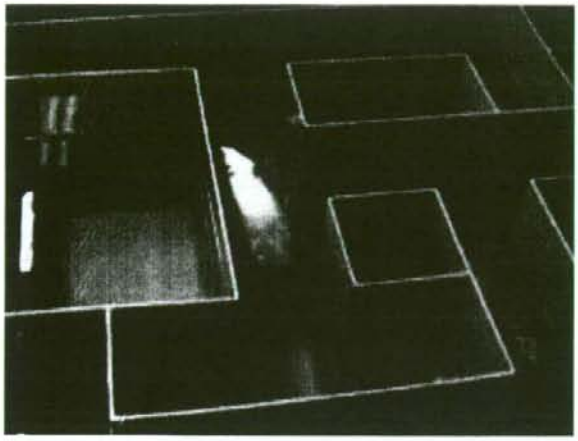

Fig. 4. Water maze test - 2
Neurocognitive outcome was assessed on the 1st, 3rd, 5th, and 7th day after CPB by testing visual-spatial learning with the maze tests (Water maze test - 2).

3．病理学的評価（海馬）

各群の海馬部においてヘマトキシリン・エオジン染色による病理学的評価を行ったが、異常所見を認めず、神経細胞数の比較においても有意差を認めなかった。

D. 考察

新生児及び乳児の先天性心奇形に対しての人工心肺技術と治療戦略は，この10年の間に急速に進化し外科的な治療成績を改善させてきた．しかしながら，全身的な炎症反応を起こすことなく人工心肺を用いることは未だ不可能であり，多くの終末臓器で多かれ少なかれ機能障害を引き起こす可能性がある．脳において，人工心肺の使用は脳血管内皮細胞機能と血流量を減少させる[9,10]．これはno-reflow現象としてよく知られており，術後の脳神経障害との関連も報告されている[11,12]．

血液希釈を可能な限り少なくするために，人工心肺充填量は回路の小型化によってかなり減少した[13-15]．しかしながら，施設間の差はあるにせよ現状ではかなり大きな乳児に対して無輸血充填が可能になっているにすぎず，それよりも小さい乳児及び新生児は種々の合併症のリスクがあるにもかかわらず同種血輸血の使用を余儀なくされている．人工心肺と同様に，同種血輸血も単独で全身の炎症性サイトカインを刺激し[16,17]，脳血流とその機能に対してリスクになりうるという報告も近年増加している[18]．

人工酸素運搬体は，このジレンマを解決するひとつの手段である．かつてFluosolが心筋の局所灌流を増やす能力を調査する為に，ブタの人工心肺モデルが用いられた[19,20]．しかしながら重篤な副作用によりFluosolの臨床使用は中止された．一方でIzumiは，イヌの急性期人工心肺モデルを用いて，同様の人工酸素運搬体が赤血球と同等の酸素運搬能を有していることを証明した[21]．HbVは他の多くのヘモグロビンに基づく酸素運搬体にみられるような微小血管の血管収縮の副作用もない．

HbVを新生児及び乳児の人工心肺の充填液に用いるためには，使用後数日の間に生じる細かな副作用を如何に少なくできるかが鍵となる．HbVをラットに使用した今までの研究で，大量のHbVを短時間で注入することが主要器官の機能を軽度ではあるが一過性に変動させることがわかっている[3,22]．したがって人工心肺運転後にmodified ultrafiltrationに準じた方法で[23]，充填液から大部分のHbVを除去することができれば，これらの潜在的な副作用は極めて少なくできると考えられた．

我々のラット人工心肺モデルにおいて，運転中のヘマトクリットはおよそ10%で，一般臨床上は輸血によって対処されるレベルのものであった．いずれの群のラットも７日間全て生存した．標準化神経学的機能試験においても有意差は認めなかった．手術７日後に犠牲死させた後に行った病理学的評価でも，３群間の所見は全て類似していた．HbV (-) prime群は，極度の血液希釈が生じており酸素供給に関して非常に不利ではあるが，標準化神経学的機能試験においては所見がなく，結果としてこの血液希釈は無症状だった．この結果は，我々のモデルが脳機能の微妙な損傷を見つける際に，非常に感度が高いことを示唆している．

学習と記憶は高次脳機能のうちの１つであり，これらを２つの迷路試験によって評価した．これらの結果，血液希釈を伴う体外循環が記憶学習機能に障害をもたらすと考えられた．人工心肺運転中における酸素供給のわずかな破綻が，他の標準化神経学的所見なしに，高次の認知機能障害のみによって発見されたことは，決して驚くべきことではない．

本研究の結果は，血液希釈を予防するためにHbVが同種血輸血の代わりに用いることができることを明らかに示している．認知機能評価の結果をふまえるとHbVの人工心肺使用は，潜在的に同種血輸血を充填液に使用するより優れていさえする可能性があった．

これらのデータの正当性を，今後より大きい動物の人工心肺モデルにて証明すると共に，脳酸素代謝と炎症反応に関してHbVの効果を評価していく必要がある．

E. 結論

ラット人工心肺モデルでのHbV使用は技術上問題なかった．このモデルにおいて，短時間の人工心肺運転であっても血液希釈を伴う体外循環は記憶学習機能に障害をもたらすと考えられた．一方HbVによる人工心肺回路充填で得られる酸素運搬量の増大が，高次脳機能の障害を軽減させる可能性が示唆された．

謝辞

HbVを提供して頂いた早稲田大学理工学総合研究センター名誉教授の土田英俊先生および関係者の皆様方に心より感謝申し上げる。本研究成果は、厚生労働科学研究費補助金により推進された。ここに記して謝意を表する。

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Artificial Cells, Blood Substitutes, and Biotechnology, 34: 1–10, 2006

ISSN: 1073-1199 print/1532-4184 online
DOI: 10.1080/00207450500428204

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Interaction of Hemoglobin Vesicles, a Cellular-Type Artificial Oxygen Carrier, with Human Plasma: Effects on Coagulation, Kallikrein-Kinin, and Complement Systems

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Abstract: Hemoglobin vesicles (HbVs), cellular-type artificial oxygen carriers containing human hemoglobin, were assessed for their biocompatibility by mixing with human plasma *in vitro*. Among three kinds of HbVs (PEG-DPEA-HbV, PEG-DPPG-HbV and DPPG-HbV), PEG-DPEA-HbV did not affect the extrinsic or intrinsic coagulation activities of the plasma, while PEG-DPPG-HbV and DPPG-HbV tended to shorten the intrinsic coagulation time. The kallikrein-kinin cascade of the plasma was slightly activated by PEG-DPPG-HbV

This study was supported, in part, by a Health Science Research Grant (Artificial Blood Project) from the Ministry of Health and Welfare, Japan, and by Grants-in-Aid for Scientific Research from the Japanese Ministry of Education, Science, Sports and Culture.

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and DPPG-HbV, but not by PEG-DPEA-HbV. The complement consumption of the plasma was observed by incubation with DPPG-HbV, but not with PEG-DPEA-HbV or PEG-DPPG-HbV. These results indicate that PEG-DPEA-HbV has a higher biocompatibility with human plasma.

Keywords: Oxygen carrier; Liposome; Hemoglobin vesicles; Complement; Kallikrein-Kinin

INTRODUCTION

Hemoglobin vesicles (HbVs) are human hemoglobin encapsulated into a lipid bilayer (*i.e.* liposome) with a polyethylene glycol (PEG) surface modification and have been developed as an artificial oxygen carrier. We have been evaluating the biocompatibility of the HbVs *in vitro* using human blood. So far, we have revealed that HbVs hardly activated platelets in terms of the aggregation response, p-selectine expression and the release of RANTES [1,2]. For neutrophils, HbVs have the least effect on the chemotactic activity, gelatinase B release, up-regulation of Mac-1 expression and superoxide production triggered by f-MLP [3]. On the basis of these experiments, HbVs appeared to have an acceptable biocompatibility to human blood.

In general, liposomes interact with various kinds of biological components *in vitro* and *in vivo*. The interaction depends on the liposome characteristics such as particle size, surface charge, lipid composition and surface modification. It is widely recognized that negatively charged liposomes activate complement in the rat, guinea pigs and humans [4–6], resulting in rapid removal from the blood circulation as opsonized liposomes by the reticuloendothelial system. A negatively charged surface triggers the intrinsic coagulation pathway and kallikrein-kinin cascade by activating coagulation factor XII (FXII) [7,8]. In addition, the cholesterol contents affects the complement activation [5,9], which is thought to be mediated by natural antibodies [10].

Incorporation of the PEG-conjugate lipid into liposomes (PEGylation) has been reported to be efficacious to avoid these biological responses [11–13]. Indeed, liposome-encapsulated hemoglobin (LEH), which was not PEGylated, has induced complement activation *via* both the classical and alternative pathways in human serum containing natural anti-phospholipid antibodies [14]. These phenomena become of great concern as a pseudoallergic reaction [15,16], which was already observed in a pig model [17]. Recently, however, it has been reported that not only LEH but also PEGylated-liposomes induce hypotension, flushing, respiratory distress, decrease of mean arterola pressure and chest pain [18,19].

In this study, we have used three modified HbVs and assessed their effects on plasma coagulation activity, the kallikrein-kinin cascade and the complement system using human blood. The results obtained here have reinforced the higher biocompatibility of HbVs.

MATERIALS AND METHODS

Liposomes

HbVs were prepared as previously described [20,21]. Briefly, hemoglobin solution prepared from outdated red blood cells for transfusion was heated under a CO gas atmosphere to inactivate possibly contaminated viruses and to remove the stroma and non-hemoglobin proteins [22]. After the removal of impurities by centrifugation and filtration, hemoglobin solution was encapsulated into liposomes by mixing with lipids. The liposomes were then extruded through membrane filters with a pore size of 0.22 μm. Three kinds of HbVs were prepared in this study and the lipid composition (mol%) was as follows: dipalmitoyl phosphatidylcholine (DPPC):cholesterol (CHOL):dipalmitoyl phosphatidylglycerol (DPPG):polyethylene glycol-conjugated distearoyl phosphatidylethanolamine (PEG5000-DSPE) = 5:5:1:0.033 (designated PEG-DPPG-HbVs); DPPC:CHOL:DPPG = 5:5:1 (DPPG-HbVs); DPPC:CHOL:dipalmitoyl-L-glutamate-*N*-succinic acid (DPEA):PEG5000-DSPE = 5:5:1:0.033 (PEG-DPEA-HbVs). All lipids were purchased from Nippon Fine Chemical Co. (Osaka, Japan) except PEG5000-DSPE, which was from NOF Co. (Tokyo, Japan). HbVs were suspended in saline and each of them contains 10 g of Hb/dL, 5.7 g lipids/dL and <0.1 endotoxin unit of lipopolysaccharide/mL. Empty liposome (Coatsome EL-A, 3.1 g lipids/dL) was purchased from NOF Co. and the lipid composition (mol%) was DPPC:CHOL:DPPG = 30:40:30. The particle size and surface charge as the zeta potential of HbVs suspended in saline were measured by Photal ELS-8000HO (OTSUKA Electronics, Tokyo, Japan).

Measurement of Coagulation Activity

Human plasma was prepared from voluntary donated whole blood at Japanese Red Cross blood centers and was stored at $-80°C$ until use. The prothrombin time (PT) and activated partial thromboplastin time (APTT) as an extrinsic and intrinsic coagulation system, respectively, were determined using a physical clotting assay on a coagulation analyzer

(KC10; Amelung, Lehbrinksweg, Germany) at several mixing ratios with plasma and liposomes. Reagents for these assays were purchased from Dade International Inc. (Miami, FL). PT and APTT were measured according to the manufacturer's instructions. Briefly, the mixtures at ratios of 20:80, 40:60 or 60:40 (v/v) of plasma:HbVs or saline were dispensed into a sample cup in duplicate. After the addition of reagents, time to coagulation was measured automatically.

Detection of Kallikrein Activation

The presence of kallikrein activation was evaluated as the degradation of high-molecular-weight kininogen (HMWK), which is substrate of kallikrein naturally existing in the plasma. HMWK was purchased from Enzyme Research Laboratories Inc. (South Bend, IN); anti-human HMWK light chain antiserum from Nordic Immunological Laboratories (Capistrano Beach, CA); plasma kallikrein from Sigma Chemical Co. (St Louis, MO). Plasma was incubated with HbVs or saline at ratios of 80:20, 60:40 or 40:60 (v/v) at 37°C for 24 h. After centrifugation at 15,000 g for 45 min at 4°C, supernatants were treated with 1% sodium dodecyl sulfate (SDS) and 1% 2-mercaptoethanol at 95°C for 10 min and then electrophoresed on a 5–20% gradient polyacrylamide gel containing 0.1% SDS. After proteins were transferred from the gel to nylon membrane, the membrane was treated primarily with anti-HMWK serum, and secondly with anti-goat IgG antibody labeled with horseradish peroxidase. HMWK was detected with a chemiluminescence detection system (ECL; Amersham, Buckinghamshire, UK). Single chain HMWK (S-HMWK) (33 μg) was digested with plasma kallikrein (6 mU) at 37°C for 2 h. Both digested and undigested S-HMWK were used as standards for Western blot analysis.

Measurement of Complement Titer

Human serum was prepared from whole blood of voluntary donors and stored at −80°C until use. Serum was incubated with HbVs, EL-A or saline at ratio of 80:20 or 60:40 (v/v) at 37°C for 1 h. After centrifugation at 15,000 g for 45 min at 4°C, supernatants were stored at −80°C until assay. The complement titer was measured using a 50% hemolysis assay based on Mayer's method with a commercial kit (New One point CH50 (KW); Japan BCG Supply Co., Tokyo, Japan). Therefore, the units of the complement titer were expressed as CH50.

Statistical Analysis

Data were analyzed with parametric Repeated Measures ANOVA following Dunnett post hock test. Statistical significance against saline in each group was established at $p < 0.05$.

RESULTS

Characteristics of Liposomes

The characteristics of HbVs and EL-A are shown in Table 1. The particle sizes of HbVs were 210–240 nm in diameter. The surface charge of PEG-DPPG-HbV and PEG-DPEA-HbV were –3.4 mV and –2.6 mV, respectively, in saline, and were considered to be neutral rather than negative. PEGylation of DPPG-HbV reduced the surface charge from –14.5 mV to –3.4 mV. EL-A, the negatively charged liposome used as a control, had a surface charge of –47.2 mV.

Coagulation Activity

Prolongation of both PT and APTT was observed as the increase of HbVs or the saline ratio to plasma (Table 2). Plasma dilution with HbVs or saline at 20% slightly prolonged or shortened the coagulation time in PT and APTT. Although a significant difference in PT was observed in three HbVs compared to saline at plasma ratios of 20% and 60%, the difference of the mean values was less than 1 second. No significant difference in APTT was observed in PEG-DPEA-HbV at any plasma ratio, but PEG-DPPG-HbV and DPPG-HbV significantly shortened the APTT. However, the shortened time was less than 2 seconds for the mean values.

Table 1. Characteristics of liposomes

Liposomes	Diameter (nm)	Zeta potential (mV)
DPPG-HbVs	239.7	−14.5
PEG-DPPG-HbVs	221.7	−3.4
PEG-DPEA-HbVs	209.9	−2.6
EL-A	162.0	−47.2

Table 2. Plasma coagulation activity as measured by PT and APTT

	Additives	Mixture ratio (additives:plasma) 0:100	20:80	40:60	60:40
PT (sec)	Saline	10.2 ± 0.17	10.6 ± 0.19	11.9 ± 0.20	15.1 ± 0.32
	DPPG-HbV	10.3 ± 0.26	10.8 ± 0.16*	11.9 ± 0.19	14.5 ± 0.27*
	PEG-DPPG-HbV	10.2 ± 0.17	10.8 ± 0.18*	11.8 ± 0.21	14.7 ± 0.25*
	PEG-DPEA-HbV	10.2 ± 0.20	10.8 ± 0.16*	11.8 ± 0.20	14.7 ± 0.25*
APTT (sec)	Saline	30.2 ± 0.63	29.3 ± 0.51	31.8 ± 0.58	40.8 ± 0.63
	DPPG-HbV	30.3 ± 0.33	28.1 ± 0.12*	31.3 ± 0.21	38.8 ± 0.37*
	PEG-DPPG-HbV	30.0 ± 0.38	27.7 ± 0.20*	30.4 ± 0.18*	38.4 ± 0.34*
	PEG-DPEA-HbV	30.0 ± 0.47	29.0 ± 0.44	31.2 ± 0.43	40.8 ± 0.63

Data are represented as the mean ± SEM using plasma from five individuals.
*Significantly different from saline ($p < 0.05$).

Kallikrein-Kinin Cascade

Degradation of HMWK was observed in the plasma incubated with DPPG-HbV and PEG-DPPG-HbV, but not with PEG-DPEA-HbV (Fig. 1). The decrease of intact HMWK and the increase of S-HMWK were evident at a ratio of 60% DPPG-HbV and PEG-DPPG-HbV.

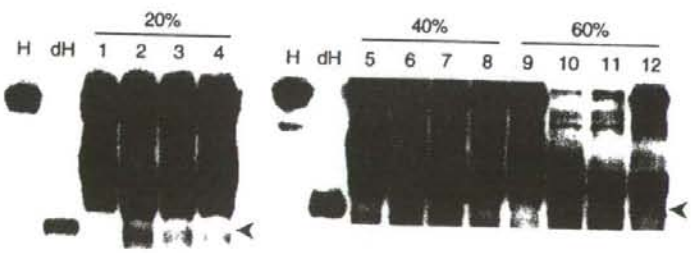

Figure 1. Activation of kallikrein-kinin cascade by HbVs. HbVs or saline were mixed with plasma as indicated ratio (v/v) at 37°C for 24 h. Appearance of digested S-HMWK was detected using western blot analysis as a result of kallikrein activation. Arrows indicate digested S-HMWK. A typical result of three independent assays is shown. H, S-HMWK; dH, digested S-HMWK; lanes 1, 5 and 9, saline; lanes 2, 6 and 10, DPPG-HbV; Lanes 3, 7 and 11, PEG-DPPG-HbV; lanes 4, 8 and 12, PEG-DPEA-HbV.

Complement Consumption

The residual complement titer in serum mixed with PEG-DPEA-HbV or saline was decreased in accordance with the dilution ratio of the additives (Fig. 2A). No difference in the complement titer was observed between

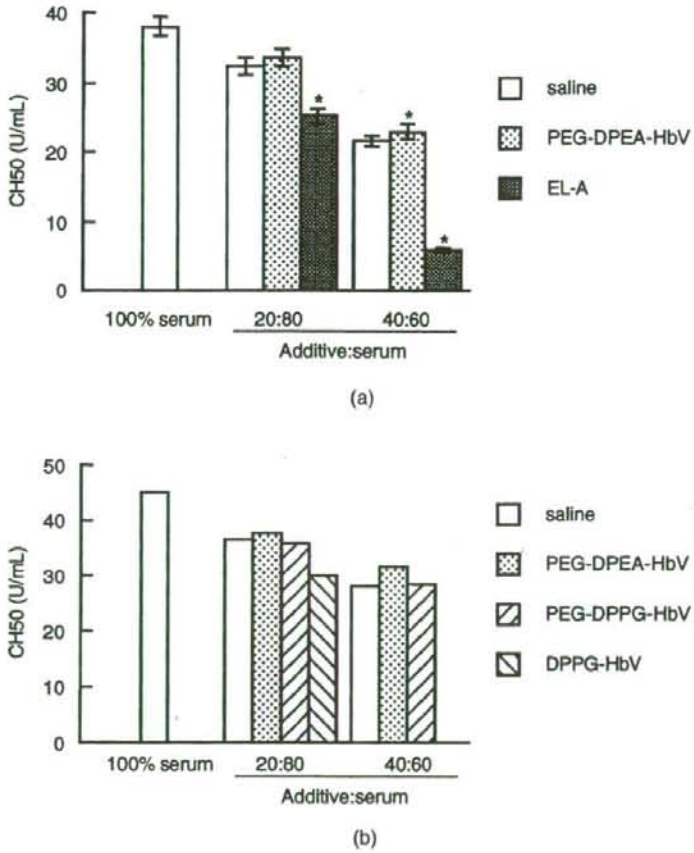

Figure 2. Consumption of complement by HbVs. HbVs, saline or EL-A were mixed with serum as indicated ratio (v/v) at 37°C for 24 h. A: Data are represented as the mean ± SEM using serum from five individuals. *Significantly different from saline ($p < 0.05$). B: Representative data are shown using one serum.

PEG-DPEA-HbV and saline, indicating that PEG-DPEA-HbV did not consume the complement component. The negatively charged liposome, EL-A, further reduced the complement titer, suggesting that the complement was activated by EL-A (Fig. 2A). No difference was observed between PEG-DPEA- and PEG-DPPG-HbV in the complement consumption (Fig. 2B). However, DPPG-HbV significantly consumed complement as no residual complement titer was detected with a mixing ratio of 40%.

DISCUSSION

We have evaluated the biocompatibility of HbVs using human plasma *in vitro*. We first investigated the effects of HbVs on the plasma coagulation activity. It is important for HbVs to have no effect on the coagulation activity of the plasma. As shown in Table 2, significant prolongations of both PT and APTT were observed as the increase of HbVs or saline ratio to plasma. However, even with the mixing ratio of HbVs or saline at 40%, PT and APTT were maintained in the normal range, 10–14 sec and 26–38 sec, respectively. Interestingly, the shortening of APTT was observed with the mixing ratio at 20% and its degree was significant for DPPG-HbV and PEG-DPPG-HbV compared to saline. The important observation was that only PEG-DPEA-HbV had no effect on APTT compared to saline at any plasma ratio. In addition, HMWK degradation was observed in the plasma incubated with PEG-DPPG-HbV and DPPG-HbV. Cleavage of HMWK reflects the activation of the kallikrein-kinin cascade and the release of bradykinin. HMWK cleavage by PEG-DPPG-HbV and DPPG-HbV may become of great concern because bradykinin is considered to be one of the causes of adverse hypotensive reactions in transfusion [23]. On the other hand, PEG-DPEA-HbV did not induce HMWK digestion. Both the intrinsic coagulation pathway and the kallikrein-kinin cascade are initiated by physical contact with FXII or FXII/HMWK with negatively charged surfaces, such as liposomes. Although PEG-DPPG-HbV has a negative charge (–3.4 mV) similar to PEG-DPEA-HbV (–2.6 mV), only PEG-DPPG-HbV affected the intrinsic coagulation system and kallikrein-kinin cascade. It is unclear as to the reason why PEG-DPPG-HbV shortened APPT and activated the kallikrein-kinin cascade. It should be noted that the lipid composition is different between PEG-DPPG-HbV and PEG-DPEA-HbV; PEG-DPPG-HbV and DPPG-HbV have a phosphate group and PEG-DPEA-HbV has a carbonyl group. It is unclear whether the difference in lipid composition affects the intrinsic coagulation system and kallikrein-kinin cascade.

Several kinds of adverse reactions have been reported in the administration of PEGylated liposomes, such as hypotension, flush, respiratory distress, decrease of mean arterola pressure and chest pain [18,19]. Similarly, the decrease of C3, B and C4 was observed in patients, suggesting complement activation by the liposome [24]. These reports suggest that PEGylation may be insufficient to avoid complement activation. In this study, however, PEGylation of DPPG-HbV dramatically decreased its reactivity to the complement (Fig. 2B). It is well known that negatively charged liposomes reduced the complement titer [4–6], and we successfully reproduced this phenomenon in this study (Fig. 2 A), indicating that our complement assay system adequately estimated the complement titer. Therefore, the discrepancy of the effect of PEGylation between our study and others was unclear and may depend on the differences regarding lipid composition, liposome size or surface charge.

In summary, PEG-DPEA-HbV has an excellent biocompatibility to human plasma regarding coagulation and kallikrein-kinin systems. Most importantly, the property of PEG-DPEA-HbV of its lacking the ability of complement activation is an essential advantage not only as an oxygen carrier, but also in the pharmaceutical field, such as in liposomal therapeutic drugs and diagnostic liposomes.

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Artificial Cells, Blood Substitutes, and Biotechnology, 35: 157–172, 2007

ISSN: 1073-1199 print/1532-4184 online
DOI: 10.1080/10731190601188224

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Effects of Hemoglobin Vesicles, a Liposomal Artificial Oxygen Carrier, on Hematological Responses, Complement and Anaphylactic Reactions in Rats

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Abstract: Hemoglobin vesicle (HbV), a liposomal oxygen carrier containing human hemoglobin, was intravenously infused into rats. After the infusion of saline, the HbV or empty vesicle (EV), numbers of red cells, leukocytes and platelets in peripheral blood were unchanged during the observation period of one week in addition to each time point among three groups. However, the lymphocyte ratio transiently decreased and the granulocyte ratio increased in the HbV and EV groups at 6 h after the infusion. Those changes returned to the initial value one day after the infusion and those were maintained for the subsequent observation period. No dramatic change was seen in the ratio of $CD4^+/CD8^+$ T cells.

A transient decrease of the complement titer was observed three days after the infusion of HbV and EV, although the consumption of complement titer was not detected in rat serum by mixing HbV or EV *in vitro*, indicating that the transient decrease of complement titer *in vivo* was not due to the consumption of complement due to the interaction with HbV or EV. Multiple infusions of HbV caused the decrease of complement titer only after the first infusion and no allergic reaction was observed. No anaphylactic shock was observed in rats

This study was supported, in part, by a Health Science Research Grant (Artificial Blood Project) from the Ministry of Health and Welfare, Japan, and by Grants-in-Aid for Scientific Research from the Japanese Ministry of Education, Science, Sports and Culture.

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administered with EV several times, while ovalbumin (OVA) sensitized rats died with symptoms of respiratory distress after the second OVA administration. These results indicate that HbV could be administered without serious clinical symptoms or adverse reactions.

Keywords: Anaphylaxis; Complement; Hemoglobin vesicle; Hematological response; Liposome

INTRODUCTION

Artificial oxygen carriers as a red cell substitute have been developed for their emergency use, capacity for long-term storage, being no requirement of cross-match and pathogen-free blood components. Hemoglobin itself, however, is not applicable as an artificial oxygen carrier because it easily dissociates from a tetramer into a dimer and exhibits nephrotoxicity [1]. In addition, hemoglobin is considered to induce vasoconstriction based on the nitric oxide (NO) scavenging mechanism at a site between endothelium cells and the vascular smooth muscle layer [2,3]. Multiple approaches have been taken to modify Hb to resolve these limitations, including chemical crosslinking to prevent dissociation, and polymerization or polyethyleneglycol (PEG) conjugation to increase its molecular mass to avoid penetration into the endothelium gap junction [1,4–9]. Recently, recombinant hemoglobin that has a lower affinity for NO has been developed and showed the absence of vasoconstriction [10]. On the other hand, the potential use of microencapsulated-hemoglobin into polymer as a cellular type of artificial oxygen carrier was reported in 1964 [4]. Since then, the encapsulation of hemoglobin into a lipid bilayer (i.e. liposome) (liposome-encapsulated hemoglobin; LEH) to make a cellular type oxygen carrier has been developed, and this type of oxygen carrier has been regarded as another approach to prevent side-effects and toxicity of Hb [11–15].

The hemoglobin vesicle (HbV) is a human hemoglobin encapsulated into liposomes with a PEG surface modification. We have revealed that HbV exhibits less interaction with platelets [16,17], neutrophils [18] and plasma proteins including complement [19] *in vitro* using human blood. HbV was revealed not to activate and consume complement *in vitro* using human serum, while a similar type of artificial oxygen carrier, LEH, did activate the complement and triggered pseudoallergic reactions [20–22]. Therefore, it is of importance to observe whether HbV affects the complement titer *in vivo*. In addition, we wished to know whether repeated administration of HbV induces an allergic or anaphylactic reaction.

Several studies showed that HbV resuscitated against hemorrhagic shock equivalent to red cell transfusion in model rats [23,24] and rabbits