総 説

ヒト赤血球由来ヘモグロビンによるヘモグロビン小胞体の開発と酸素輸液としての医療応用

Development and Clinical Application of Hemoglobin Vesicles as an Oxygen Carrier Using Hemoglobin Derived from Human Red Blood Cells

高木智史　大村光代　太田勝次　須賀裕子　松浦昭宏

Eugene Satoshi Takagi, Mitsuyo Ohmura, Katsuji Ohta, Yuko Suka, Akihiro Matsuura

和文抄録

日本赤十字社より供与された期限切れヒト赤血球より精製，ウィルス除去したヘモグロビンをリポソーム脂質二重膜に内包したヘモグロビン小胞体（HbV）は，ヒト赤血球と比較し粒径は約1/30と小さいが，同等の酸素運搬能を有することがHbVの物理化学実験および種々の実験モデルにおいて証明された．また従来のリポソーム処方で問題とされる血小板活性化，肺臓や腎臓への蓄積等の生体不適合の問題もないことが判明し，さらにラット，サルを用いた安全性試験においても特記すべき副作用は見られなかった．これらHbVの生体内における機能と安全性の評価より，産学協同の国家プロジェクトとして臨床応用を目指した開発が鋭意進行中である．

Abstract

Hemoglobin vesicle (HbV) is prepared by encapsulating virus-free hemoglobin, which is purified from outdated human red blood cells (RBCs) provided from Japanese Red Cross, into liposome with a phopholipid bilayer membrane. HbV with its diameter approximately one-thirtyth smaller than that of RBC is proved to be effective as an oxygen carrier comparable to human red blood cells as evidenced by various physicochemical and pharmacological experiments. Animal studies also demonstrated that HbV was biologically conformable without platelet activation, accumulation in pulmonary and renal tissues and so on, which have been recognized as problems of conventional liposomes, and that HbV caused no notable adverse effect in safety studies using rats and monkeys. Based on these evidences, the research and the development of HbV aiming at its clinical application is intensively in progress as a national project by industry-university cooperation.

Keywords

hemoglobin vesicle, liposome, human hemoglobin, resuscitation, exchange transfusion, safety study

1980年代より早稲田大学理工学部の土田，武岡らによって始められた人工酸素運搬体（ヘモグロビン小胞体；HbV）の開発は，1990年代に慶應義塾大学医学部の小林，末松らがさらに参画し，処方の最適化，製剤規格の設定がなされ，効果・安全性も確認された．2002年より両大学からstart-upした株式会社オキシジェニクスも加わり，これまで両大学の共同研究で蓄積された膨大な技術，データを基に医薬品としての実用化に向けた舵取りがなされた．すなわち，HbVの形状，ヘモグロビン純度等の物性データ，安全性，体内滞留性，体内動態，酸素運搬能等の生物学的データより，単回投与での救命救急の酸素輸液としての実用化の目途が立ったと判断された．本総説ではHbVの概略と医療応用に向けた最近の知見について述べる．

1．HbVの概念

ヒト赤血球の直径は約8 μmであり，直径が4 μmの毛細血管内を変形して通過し，末梢組織でのガス交換を司る．ヒト赤血球の寿命は約120日と言われているが，老化するに従って赤血球膜のintegrityは失われ，変形能は低下する．一方HbVは日本

株式会社オキシジェニクス　〒105-0001　東京都港区虎ノ門4-1-1　虎ノ門パストラル本館6階　Oxygenix Co., Ltd.　Toranomon Pastoral 6F, 4-1-1, Toranomon, Minato, Tokyo 105-0001

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赤十字社より供与された期限切れヒト赤血球から精製したヘモグロビンを内包した脂質二重膜構造から成る小胞体で，変形能は有さないが直径250nmと赤血球の約1/30と小さいために毛細管内をスムーズに通過できる（Fig. 1.）[1,2]．HbVの内水相のヘモグロビン濃度は35 g/dL，脂質被膜厚は5-10nmとヒト赤血球とほぼ同等である．HbVは膜糖鎖を有していないため，ヒト赤血球で懸念される血液型不適合の問題もなく，また包含するヘモグロビンは熱処理によりウィルスを不活性化・除去しているため[3,4]，HIVや肝炎ウィルスは感染のリスクはほとんどなく，未知の感染症に関しても現行の輸血と比較してリスクは極めて少ないと考えられる．さらにはヒト赤血球が4℃においても保存期間は最長21日間に対し，HbVは室温で1年以上の保存が可能である[1,5]．

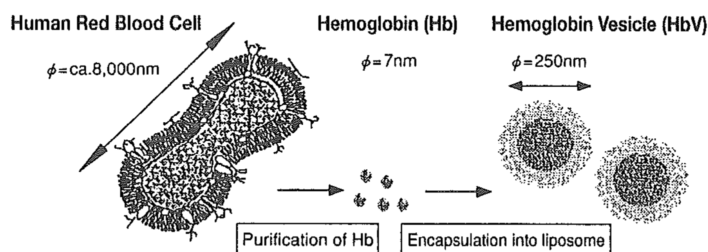

Fig. 1. Schematic representation of hemoglobin vesicle (HbV) prepared from human red blood cells

一方，ヘモグロビンの分子内架橋体や重合体，あるいはヘモグロビン表面に高分子を結合させた修飾ヘモグロビンの開発は米国を中心に行われており，その内のいくつかは臨床試験が進行している[6-9]．しかしながら粒径が数nmと微小であり，またヘモグロビンが露出していることから，fenestrationを通しての血管外への漏出やヘモグロビンにより一酸化窒素（NO）がトラップされた結果生じると考えられる血管収縮等の副作用が問題となっている．それに比較してHbVは，先に述べたように直径が大きく，また赤血球同様ヘモグロビンは脂質膜に内包されているためこのような副作用の懸念はない．

2．HbVの機能

人工酸素運搬体としてのHbVの最も，且つ唯一の機能は酸素運搬能である．HbVの酸素飽和度が50%時の酸素分圧（P_{50}）は27-34 Torrであり，ヒト赤血球のP_{50} 26-28 Torrと非常に近似している[1,10]．実験動物におけるHbVの効果はラットおよびイヌで確認されている．

1）ラット50%出血モデル：麻酔下ラットの右総頚動脈より50%の血液（ca. 28mL/kg）を 1 mL/minの速度で7-8 minかけて脱血し，15分放置後遺伝子組み換え型ヒト血漿アルブミン（rHSA）に分散したHbV，あるいはrHSAを出血量と同量，右総頚静脈より右心房へ挿入したカテーテルより5分間かけて投与し，非処置群と比較した．輸血を全く施さなかった非処置群では8例全例が死亡したのに対し，rHSA単独群では処置6時間後の死亡率は25%（2/8），HbVでは0%であった（Fig. 2.）[11]．このことよりHbVは，非処置やrHSA単独投与を上回る蘇生効果を示し，効率的な酸素運搬能が示唆された．

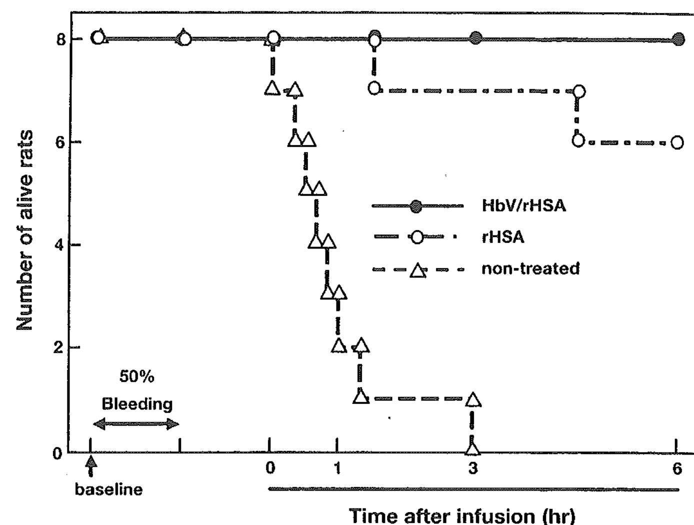

Fig. 2. Resuscitation by hemoglobin vesicle (HbV) in Wistar rats subjected to 50% hemorrhage shock.
Infusion of HbV suspended in recombinant human serum albumin (rHSA) or rHSA was started at 15 min after the 50% bleeding, and then mortality was recorded for 6 hrs. The non-treated group did not receive a resuscitative fluid after the hemorrhage. Eight rats were used for each group. (cited from Crit Care Med 2004;32:539-545)

2）ラット90%交換輸血モデル：麻酔下ラットの右総頚動脈より2 mL/minの速度で脱血し，同時に右総頚静脈より右心房へ挿入したカテーテルを通して同速度でHbV（rHSAに分散）あるいはrHSAを投与し，90%の交換輸血となるまで継続した．rHSA群では，交換輸血に伴い顕著な血圧低下ならびに腎皮質酸素分圧低下が見られたのに対し，HbV群では血圧および腎皮質酸素分圧とも低下は見られず，また心拍数，下腹大動脈血流量，血液ガス分析値等も良好に維持された（Fig. 3.）[1,12-14]．このことからもHbVは優れた酸素運搬能を有することが示唆された．

3）体内有効HbV：カニクイザルにHbV 10 mL/kg静脈内投与し，血漿中のHbV由来の総ヘモグロビンおよび非メト化ヘモグロビンを測定した．両ヘモグロビンとも経時的に低下したが，非メト化ヘモグロビンの消失半減期は約15時間と見積もられ，救命救急臨床に充分なポテンシャルを有していることが判明した[15]．

3．HbVの生体適合性

リポソーム処方の大きな問題点の一つとして，血小板を活性化し，凝集を惹起することがあげられる．これはリポソームの

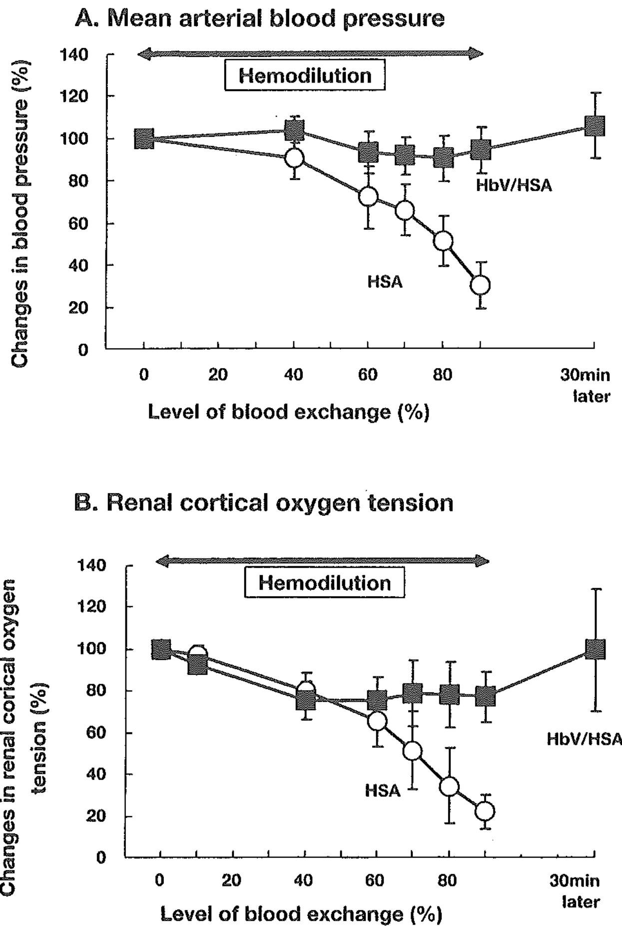

Fig. 3. HbV preserved arterial blood pressure and renal cortical oxygen tension depressed in Wistar rats subjected to 90% exchange transfusion
Ninety percent of estimated total circulating volume of blood (56 mL/kg) was exchanged with 5% human serum albumin (HSA, n=6) or HbV suspended in HSA (HbV/HSA, n=6) at 2mL withdrawal/infusion cycles at a rate of 2mL/min. Blood pressure was monitored by a pressure transducer connected to a catheter inserted into the common carotid artery. Renal cortical oxygen tension was measured by a needle polargraphic oxygen electrode placed in the cortex of the left kidney. (cited from ASAIO Journal 1997;43:289-297, Bioconjugate Chem 1997;8:23-30, Artif Cells Blood Substitutes Immobilization Biotechnol 1997;25:357-366)

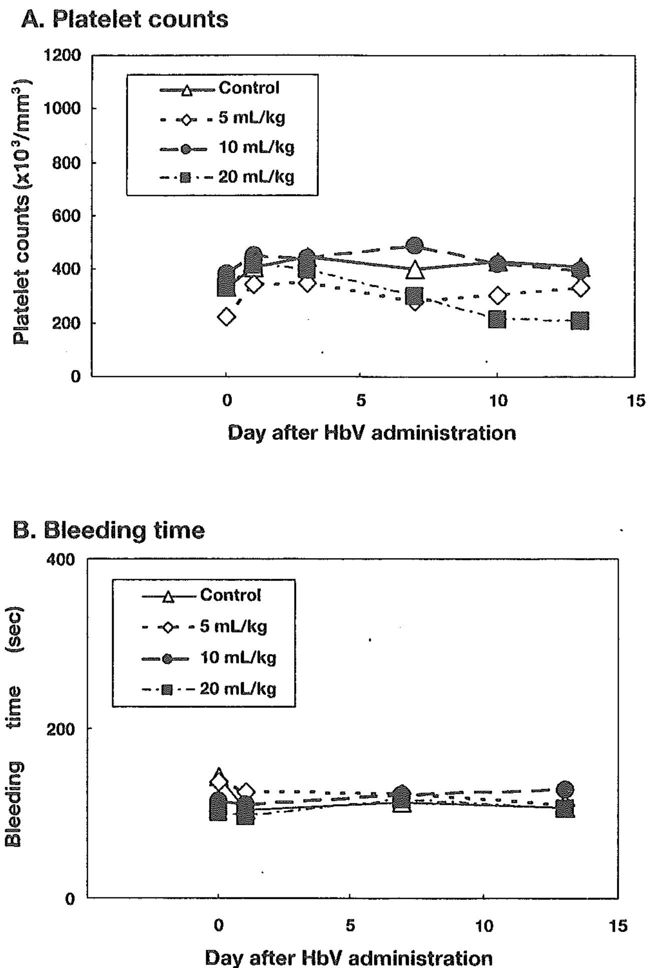

Fig. 4. Platelet counts and bleeding time in rats treated with single HbV administration in cynomolgus monkeys.
Intravenous infusion of HbV was carried out at doses of 5, 10, and 20 mL/kg (5 mL/min), and then venous platelet count and bleeding time according to Simplate method were measured. Three animals were used for each group.

脂質二重膜の構成成分に関係する．HbVでは血小板を活性化させない新脂質成分として 1,5-*O*-Dihexadecyl-*N*-succinyl-L-glutamate（DHSG）を配合し[11]，ラットおよびカニクイザルにおいてその有用性が実証された．すなわちラットにおいて各種リポソームを2 mL/bodyの投与量で投与し40分後の静脈血中の血小板数を測定した結果，他の一般的なリポソーム成分構成では血小板が約20-50%減少したのに対し，HbVでは全く減少を示さなかった[16]．カニクイザルにHbV 5，10，20 mL/kgの単回静脈投与後の血小板数は，投与後２週間にわたって背景値内で推移し，また出血時間の変動も殆ど認められなかった（Fig. 4.）[15]．更には一般的なリポソームがトラップされることが知られている肺臓，および修飾ヘモグロビンの毒性が見られる腎臓の試験終了後の組織剖検においても，HbV 5，10，20 mL/kgの投与による変化は特に認められなかった[15]．以上より，HbVの生体適合性については問題ないと判断される．

４．HbVの安全性および生体内運命

Wistar系雄性ラットにHbVを20 mL/kgの投与量で尾静脈より投与した結果，脾臓の重量（比体重）の増加，血清アルブミンの減少等が見られたがいずれも軽微であった．血清中コレステロールは経時的に増加し，投与２日後に最高値となり，その後漸次減少し１週間後には前値に復した（Fig. 5.）[17]．他には特筆する変化は認められなかった．

カニクイザルにHbVを5，10および20 mL/kgの投与量で単回静脈内投与し，投与後の血漿中のHbV由来ヘモグロビン（HbV-Hb）濃度推移を２週間にわたって観察した．いずれの

投与においても血漿中のHbV-Hbは経時的に低下し，最終消失相の半減期は10 mL/kgで2.8日，20 mL/kgで2.9日であった．脂質代謝に関し，血清の総コレステロールは10 mL/kgおよび20 mL/kg投与群で投与後7日および13日で上昇していた．また5 mL/kg/dayおよび10 mL/kg/dayの7日間反復投与においても，血清コレステロールは8日目以降上昇していた[15]．これらラットおよびサルで見られた血清コレステロールの増加は体内で代謝されたHbVのリポソーム成分によるものと考えられた．今後精査を要する．

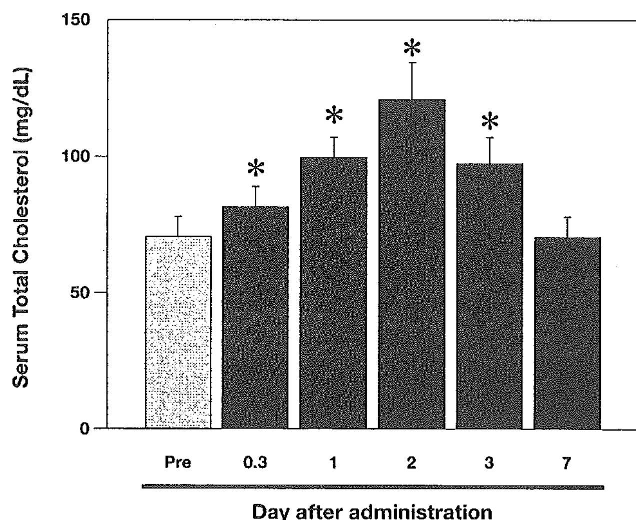

Fig. 5. Changes in serum total cholesterol after single intravenous administration of HbV in rats.*; significantly different from pre-value ($p<0.05$)
HbV was intravenously administered in Wistar rats (n=5) at a dose of 20 mL/kg, and serum cholesterol level was measured 0, 0.3, 1, 2, 3, 7 days after the administration.(cited from Biomaterials 2004;25:4317-4325)

5．臨床応用に向けた取り組み

2004年秋に米国食品医薬品局（FDA）より試案として赤血球代替物のガイダンス（Criteria for Safety and Efficacy Evaluation of Oxygen Therapeutics as Red Blood Cell Substitute）が出された[8]．それによると，赤血球代替物による酸素治療の潜在的な利点は，1）万能な適合性，2）緊急使用，および3）長期保存であるとしている．さらに「赤血球代替物による酸素治療の有効性および安全性検討のための指標」ならびに「リスクとその利点評価」のための臨床試験デザインに関するガイダンスが示され，適用として1）局所的酸素供給（心臓血管手術時灌流，放射線治療あるいは化学療法時の腫瘍の感受性増強など），2）手術中およびその前後の適用（待機的外科手術），3）外傷，をあげている（Table 1.）．また赤血球代替物による酸素治療薬は最優先審査承認制度（Fast Track）の対象になるとされている．まだ草稿段階とはいえ，FDAがこのようなガイダンスを出したということにより，人工血液開発に関し具体的な指針と新たな局面を与えたと判断される．ガイダンスには，人工血液はOxygen Therapeuticと表記されていることにより，血液代替物として血液と比較しての存在ではなく，酸素治療薬としての新しい存在意義を獲得したと考えられる．

Table 1. Potential indications for oxygen carriers*

1. Local Effects/Regional Perfusion
1) Perfusion during coronary angioplastry
2) Increaseing the sensitivity of tumors to radiation or to chemotherapy
2. Perioperative Indications
1) Elective surgical indications
3. Trauma

*; U.S. Food and Drug Administration. Draft Guidance for Industry: Criteria for Safety and Efficacy Evaluation of Oxygen Therapeutics as Red Blood Cell Substitutes - 10/28/2004

人工血液としては，米国Northfield Laboratories社（http://www. northfieldlabs. com）のヒト由来ヘモグロビンの重合体を製剤化したPolyHeme®の開発が進行している．救命救急での出血性ショックを対象とし，現在第Ⅲ相臨床試験を実施している．生理食塩液を対照薬として720名の重篤な出血性ショック患者に盲検的に救急車内＋病院到着後12時間まで投与し，エンドポイントとしてその後30日間フォローアップするというものである．本臨床試験では，救命救急に用いられるということで一般的なインフォームド・コンセントの適用は除外された．

このように人工血液を取り巻く状況の変化の中で，HbVは世界初のセル型人工酸素運搬体として位置づけを確保すべく，臨床応用への取り組みが進行している。先に述べたHbVの生体内における機能と安全性の評価より，実用化に向けたHbVの製剤処方が決定された．同時にHbV製剤の規格・試験法の設定とともに，製造規模の拡大の検討がすでに開始された．1990年代初めには1バッチあたり数十mLであったが，文部科学省，厚生労働省の科学研究費助成によって量産化技術が進展し，2002年には2L/batchと，またHb精製量も4L/batchと大幅に向上したことよりGMPスケールアップが可能と判断された．そこで早稲田大学の指導のもと，ニプロ株式会社と協力して工業化を推し進めている．すなわちオキシジェニクス社京都研究所において早稲田大学からの技術移転により工業化を目指した小規模製造を開始し，さらにニプロ社により大規模製造のための工業化検討を行う予定である．また早期の臨床応用を目指して適応症の絞込みも行われ，2006年に臨床研究を開始すべく順調に準備が進んでいる．

早稲田·慶應義塾大学の共同研究から技術確立されたヒトヘモグロビン小胞体は，オキシジェニクス社による非臨床試験・臨床試験およびパイロットスケールの製造，ニプロ社による治験薬・最終製品の製造という役割分担の中，産学協同の国家プロジェクトとして，また国際展開についても視野に入れ，早期の臨床応用を目指して開発を行っている．

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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

Hiromi Sakai, Hirohisa Horinouchi, Manabu Yamamoto, Eiji Ikeda, Shinji Takeoka, Masuhiko Takaori, Eishun Tsuchida, and Koichi Kobayashi

BACKGROUND: Hemoglobin-vesicles (HbVs; diameter, 251 ± 81 nm) are artificial O_2 carriers. Their efficacy for acute exchange transfusion has been characterized in animal models. However subsequent profiles of recovery involving the degradation of HbV in the reticuloendothelial system (RES) and hematopoiesis remain unknown.
STUDY DESIGN AND METHODS: Isovolemic 40 percent exchange transfusion was performed in 60 male Wistar rats with HbV suspended in 5 g per dL recombinant human serum albumin (rHSA; HbV/rHSA, [Hb] = 8.6 g/dL), stored rat RBCs suspended in rHSA (sRBC/rHSA), or rHSA alone. Hematological and plasma biochemical analyses and histopathological examination focusing on the spleen were conducted for the subsequent 14 days.
RESULTS: The reduced hematocrit (Hct) level (26%) for the HbV/rHSA and rHSA groups returned to its original level (43%) in 7 days. Plasma erythropoietin was elevated in all groups: the rHSA group showed the highest value on Day 1 (321 ± 123 mIU/mL) relating to the anemic conditions (HbV/rHSA, 153 ± 22; sRBC/rHSA, 63 ± 7; baseline, 21 ± 3). Simultaneously, splenomegaly occurred in all the groups as HbV/rHSA > rHSA > sRBC/rHSA. Histopathologically, the accumulated HbV in the spleen was undetectable by Day 14, but hemosiderin was deposited in slight quantities for both the HbV/rHSA and sRBC/rHSA groups. Considerable amounts of erythroblasts were apparent in the spleens of both the rHSA and the HbV/rHSA groups.
CONCLUSION: HbVs were phagocytized and degraded in RES, a physiological compartment for the degradation of RBCs, and the elevated erythropoietic activity resulted in the complete recovery of Hct within 7 days in the rat model.

Hemoglobin (Hb)-based O_2 carriers (HBOCs) have been developed progressively for use as a transfusion alternative. Some are now undergoing clinical trials.[1,2] Advantages of HBOCs include the absence of blood-type antigenicity and infectious pathogens and stability for long-term storage when compared with RBC transfusion.[3] Considerably shorter half-life ($t_{1/2}$) of the HBOCs in the blood stream (2-3 days) limit their use,[4] but they are applicable for shorter periods of use as: 1) a resuscitative fluid for hemorrhagic shock during an emergency situation temporarily or for bridging until RBCs are available;[5] 2) a fluid for preoperative hemodilution or perioperative O_2 supply fluid for a hemorrhage during elective surgery to avoid or delay alloge-

ABBREVIATIONS: HBOC(s) = hemoglobin-based O_2 carrier(s); HbV(s) = hemoglobin-vesicle(s); MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration; PLP = pyridoxal 5′-phosphate; RES = reticuloendothelial system; rHSA = recombinant human serum albumin; sRBC(s) = stored red blood cell(s).

From the Advanced Research Institute for Science and Engineering, Waseda University, Tokyo; the Departments of General Thoracic Surgery and Pathology, School of Medicine, Keio University, Tokyo; and the East Takarazuka Satoh Hospital, Takarazuka, Japan.

Address reprint requests to: Koichi Kobayashi, Department of General Thoracic Surgery, School of Medicine, Keio University, Tokyo 160-8582, Japan; e-mail: kobayash@sc.itc.keio.ac.jp.

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neic transfusion;[6] 3) a priming solution for the circuit of an extracorporeal membrane oxygenator during cardiac surgery;[7] and 4) an alternative for use for other potential indications, for example, so-called O_2 therapeutics to oxygenate ischemic tissues.[8,9]

A phospholipid vesicle or liposome-encapsulating concentrated human Hb (Hb-vesicle, HbV) is an HBOC.[10,11] The cellular structure of the HbV (particle diameter, approx. 250 nm) has characteristics that resemble those of natural RBCs because both have lipid bilayer membranes that prevent the direct contact of Hb with blood components and the endothelial lining, thus shielding all side effects of molecular Hb.[12,13] Once in circulation, HbV particles are captured by the phagocytes in the reticuloendothelial system (RES or mononuclear phagocytic system) and are metabolized in the physiologically normal pathway after topload infusions.[14-17]

We tested the efficacy of HbV suspended in plasma-derived and recombinant human serum albumin (rHSA) in extreme normovolemic hemodilution (80-90% blood exchange) and resuscitation from hemorrhagic shock. They have a comparable O_2-transporting capacity with RBCs.[18-21] However, only a few hours of observation after extensive blood exchange has been reported.

This study undertakes, for the first time, a longer period of observation (2 weeks) after moderate and clinically relevant isovolemic exchange transfusion of a 40 percent estimated blood volume with HbV suspended in a 5 g per dL rHSA solution.[21] We analyzed plasma biochemical, hematological, and histopathological examinations, particularly addressing the degradation of HbV in RES and erythropoietic activity after the reduced Hct. Splenomegaly was more dominant than hepatomegaly after single and repeated infusions of HbV in our previous studies.[14,15,17] Senescent RBCs are known to be captured and degraded in the spleen.[22] For that reason, we conducted infusion of stored homologous RBCs to compare the relative impacts on the spleen.

MATERIALS AND METHODS

Preparation of HbVs suspended in rHSA

HbVs were prepared under sterile conditions, as reported in previous studies.[23,24] The Hb was purified from outdated donated blood provided by the Japanese Red Cross Society (Tokyo, Japan). The encapsulated Hb (38 g/dL) contained 14.7 mmol per L pyridoxal 5′-phosphate (PLP) (Sigma-Aldrich Corp., St. Louis, MO) as an allosteric effector at a molar ratio of PLP/Hb of 2.5. The lipid bilayer comprised 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine, cholesterol, 1,5-*O*-dihexadecyl-*N*-succinyl-L-glutamate (Nippon Fine Chemical Co. Ltd, Osaka, Japan), and 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-PEG_{5000} (NOF Corp., Tokyo, Japan), at a molar composition of 5/5/1/0.033. The lipopolysaccharide content, measured with a modified *Limulus* amebocyte lysate test, was less than 0.1 EU per mL.[25] The physicochemical parameters are P_{50}, 27 Torr; 251 ± 81-nm particle diameter; and less than 3 percent MetHb content. Before use, the HbV suspension ([Hb] = 10 g/dL, 8.6 mL) was mixed with a solution of rHSA (25 g/dL, 1.4 mL; Nipro Corp. Osaka, Japan) to regulate the rHSA concentration in the suspending medium to 5 g per dL. Consequently, the Hb concentration became 8.6 g/dL.[21] Under these conditions, the colloid osmotic pressure and the viscosity (300/sec, 37°C) of the HbV/rHSA were 20 mmHg and 2.9 cP, respectively.

Preparation of stored homologous RBC suspended in rHSA

Blood was withdrawn from donor Wistar rats via the caudal vena cava during ether anesthesia. This was mixed with an RBC preservation fluid, CPDA-1 (C.A. Karmi, Kawasumi Laboratories Inc., Tokyo, Japan) at the volume ratio of 10 percent. The mixture was stored under sterile conditions at 4°C for 1 week because rat RBCs stored for 1 week are reportedly as fragile as the human RBC stored for 4 weeks.[26] After preservation, the stored blood was centrifuged for 10 min at 4000 × g, and then the supernatant and the buffy coat were removed. The sedimented RBCs were resuspended in saline and centrifuged. This procedure was repeated twice. Finally, the RBCs were suspended in a 5 g per dL rHSA solution to prepare stored homologous RBCs suspended in rHSA (sRBC/rHSA). The Hb concentration was regulated at 8.6 g per dL, the same Hb concentration of HbV/rHSA.

Exchange transfusion and 2-week observations

Experiments were conducted with 65 male Wistar rats (223 ± 20 g body weight; Saitama Experimental Animals Supply Co., Kawagoe, Japan). During cannulation and exchange transfusion, the rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (1 mL/kg; Abbott Laboratories, North Chicago, IL). Polyethylene catheters were introduced into the right common carotid artery. Blood withdrawal and sample injection were repeated through one line at 1 mL per 30 seconds. Samples were HbV/rHSA (n = 20), sRBC/rHSA (n = 20), and rHSA only (n = 20). Five rats were used for baseline measurements.

The systemic blood volume was estimated to be 56 mL per kg of the total body weight.[27] Blood was exchanged under the assumption of normovolemia. Therefore, to estimate the necessary amount of HbV, the exchange was assumed to consist of repeating the number of cycles of 1.0-mL withdrawal and sample infusion. The level of exchange, 40 percent, is therefore given as

$$40\% = 100 \times \{1 - [(0.056 \times \text{body weight} - 1.0)/(0.056 \times \text{body weight})]^n\}. \quad (1)$$

The volume exchanged was calculated as $n \times 1.0$ (mL).[28] The sample volume is calculated as 6.0 mL for a rat body weight of 220 g.

After the blood exchange, the catheter was removed, the artery was ligated, and the neck skin was sutured with a stitch. The rats were housed in cages in a barrier room at the animal experimental facility of Keio University. Rats were provided ad libitum access to food and water in a temperature-controlled environment with a 12-hour dark-light cycle.

Five rats were selected randomly from each group at 1, 3, 7, and 14 days for sequential measurements. At each time point, the rats were anesthetized with a 1.5 percent sevoflurane-mixed air inhalation. After measuring the body weight, approximately 150 µL of blood was withdrawn from the tail vein via an indwelling needle (24-gauge; Nipro Corp.) for Hct measurement with glass capillaries, and blood cell counts with an automatic blood cell counter (Model KX-21, Sysmex Corp., Kobe, Japan). The animals were laparotomized and approximately 6 mL of blood was withdrawn from the caudal vena cava for the plasma biochemical tests. The organs were resected en bloc and fixed in a 10 percent formalin neutral buffer solution (Wako Pure Chemical Industries Ltd., Tokyo, Japan) and then embedded in paraffin. Four-micrometer sections were stained with the hematoxylin-eosin, Berlin blue, and Giemsa methods.

The collected blood (approx. 6 mL) was centrifuged (5,000 × *g*, 10 min) to separate the plasma, which was then ultracentrifuged (50,000 × *g*, 20 min) to sediment the HbV particles from the plasma at 1 and 3 days after the exchange transfusion with HbV/rHSA to avoid their interference by HbV particles in the plasma biochemical assays.[29] The obtained transparent serum specimens contained no Hb, indicating that no hemolysis of HbV occurred. They were stored at –80°C until biochemical tests at BML, Inc. (Kawagoe, Japan). Erythropoietin (EPO) was measured with radioimmunoassay. Because the rat EPO shows a high degree of homology with human EPO, the rat EPO cross-reacts in the assay of the antihuman EPO.[30]

The experimental protocol was fully approved by the Laboratory Animal Care and Use Committee of School of Medicine, Keio University. It also complied with the *Guide for the Care and Use of Laboratory Animals*.[31]

Statistical analyses

Data are reported as mean ± standard deviation (SD) for all measurements. Differences between the control (baseline) group and a treatment group were analyzed with a one-way analysis of variance followed by Fisher's protected least significant difference test. The changes were considered significant if the p value was less than 0.01.

RESULTS

Body and spleen weights and hematological tests

Rats of all groups tolerated well the 40 percent blood exchange; they survived until their intentional euthanization. The rats survived this intervention because of the normovolemic exchange transfusion while maintaining the blood colloid osmotic pressure with 5 g per dL rHSA as the suspending medium. All rats gained weight until their euthanization (Fig. 1). No noticeable change occurred in their behavior or appearance such as the pilomotor response.

The spleen:body weight ratio increased significantly for the HbV/rHSA group at 1 and 3 days after the exchange. It returned to a level that was comparable to the baseline at 14 days. The rHSA group also showed significant splenomegaly at 3 days, but no splenomegaly at 1 day. At 14 days, the spleen weight reverted to the baseline level. The sRBC/rHSA group also showed moderate, but significant, splenomegaly on Days 1, 3, and 7.

The Hct before the exchange transfusion was approximately 43 percent. It decreased to about 26 percent for the HbV/rHSA and rHSA groups. Both groups showed a monotonic Hct increase; at 7 days, the Hct showed a complete recovery to the baseline level (about 43%) and an overshooting at 14 days (approx. 46%). In the sRBC/rHSA group, the Hct level at 1 day was much higher than that of the other groups because of the sRBC infusion. The Hct level, however, was slightly lower than for the other groups at 7 and 14 days. The mean corpuscular Hb (MCH), mean corpuscular volume (MCV), and mean corpuscular Hb concentration (MCHC) values remained within normal ranges (data not shown); however, MCH and MCHC of the HbV/rHSA group at 1 and 3 days were not measured because of the presence of HbV. The sRBC/rHSA group showed slightly lowered MCV and MCH levels at 1 day. In contrast to Hct, platelet and white blood cell counts showed nonsignificant decreases at 1 day and then maintained rather steady values. The plasma Hb concentration derived from HbV after the exchange transfusion was estimated as 4.4 g per dL, which decreased, respectively, to 1.8 ± 0.1, 1.1 ± 0.1, and 0 g per dL on Days 1, 3, and 7.

Plasma biochemical tests

The plasma EPO level, an indicator of an anemic, hypoxic, or stressed condition, increased significantly from 21 ± 3 IU per L in the normal condition to 312 ± 123 IU per L for the rHSA group at 1 day, which was significantly higher than for the HbV/rHSA group (153 ± 22 IU/L) or the sRBC/rHSA group (63 ± 7 IU/L; Fig. 2). After 3 days, they decreased to less than 100 IU/L; at 7 days, they reverted to the baseline level.

Regarding the other routine analytes, apartase aminotransferase showed slight increases on Day 1 for all

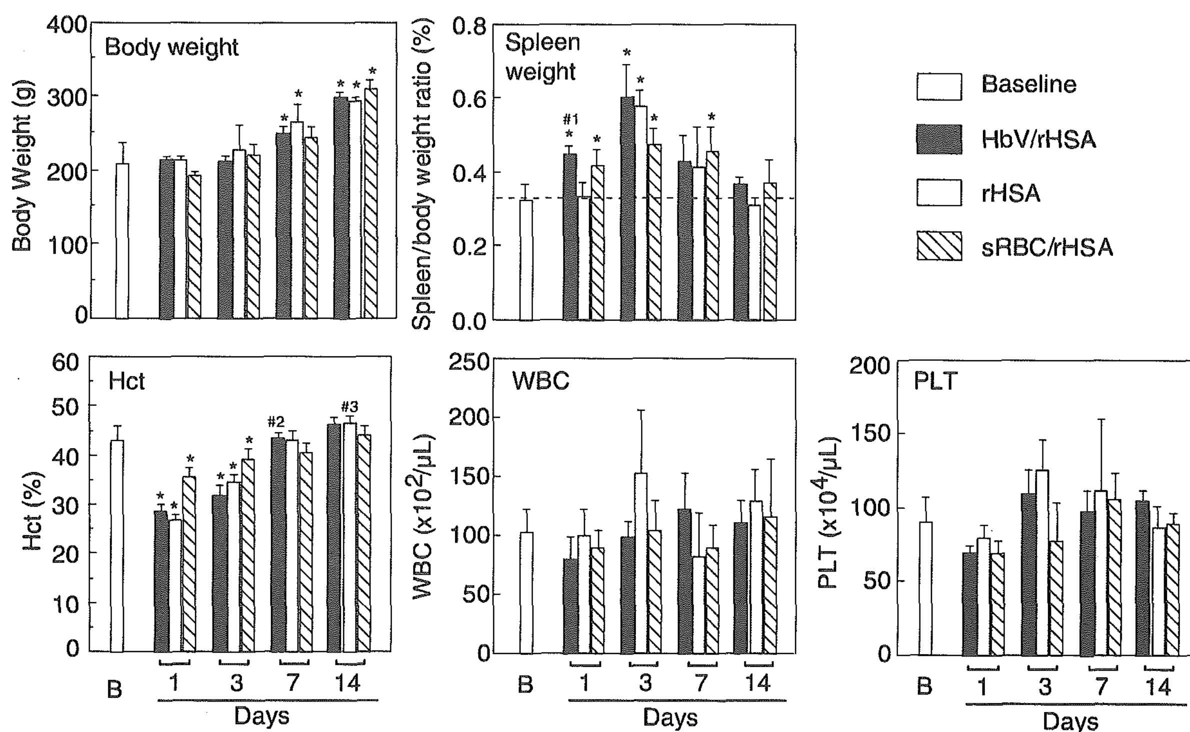

Fig. 1. Changes in body weight, spleen:body weight ratio, and hematological parameters after 40 percent exchange transfusion with HbV/rHSA, rHSA, or sRBC/rHSA. The spleen:body weight ratio (baseline, 0.32 ± 0.04%) increased significantly for the HbV/rHSA group at 1 day (0.45 ± 0.03%) and 3 days (0.60 ± 0.09%). It returned to the baseline at 14 days (0.37 ± 0.02%). The rHSA group also showed significant splenomegaly at 3 days (0.58 ± 0.05%) and returned to 0.31 ± 0.02 percent at 14 days. The sRBC/rHSA group also showed splenomegaly at 1, 3, and 7 days (0.42 ± 0.04, 0.48 ± 0.04, and 0.46 ± 0.06%, respectively). The baseline Hct level was 43 percent; it decreased to about 26 percent for the HbV/rHSA and rHSA groups. At 7 days, they showed complete recovery to approximately 43 percent and then further increased to approximately 46 percent at 14 days. The values are means ± SD. The broken line indicates the baseline value. *Significantly different from the baseline ($p < 0.01$); #1significantly different from the rHSA group ($p < 0.01$); #2p = 0.0288 versus sRBC/rHSA; #3p = 0.0353 versus sRBC/rHSA. B = baseline.

groups (HbV/rHSA, 70 ± 5 U/L; rHSA, 69 ± 12; sRBC/rHSA, 72 ± 9; baseline, 60 ± 7), but it reverted to the original level, whereas alanine aminotransferase was stable. Alkaline phosphatase and γ-glutamyltransferase showed significant or nonsignificant reductions for all groups throughout the experiment. Creatine phosphokinase was stable for 14 days. For all groups, creatinine and uric acid were maintained at low levels for 14 days (data not shown). Amylase showed some significant reduction, but did not change markedly for 14 days (Fig. 3). In contrast, lipase showed significant and marked increases for the HbV/rHSA group for 3 days, but it tended to decrease after 7 days.

Regarding plasma lipid components in the HbV/rHSA group, the total cholesterol and free cholesterol showed significant increases with maximum values at 3 days (Fig. 3). Nevertheless, they returned to their original levels at 7 days. The β-lipoprotein tended to decrease after the exchange transfusion, showing significant reductions at 3 and 7 days for the rHSA group. The high-density lipoprotein cholesterol also tended to decrease with a significant reduction at 3 days for the rHSA group. Triglyceride tended to decrease for all groups with a significant difference in the HbV/rHSA group at 1 and 3 days, partly because of ultracentrifugation of the plasma fractions, and in the rHSA group at 7 days. At 14 days, they generally recovered to the baseline level. The phospholipid tended to decrease with significant differences for all groups. Free fatty acid tended to increase at 14 days. The serum bilirubin (<0.1 mg/dL) remained at a low level throughout the experiment. Fe^{3+} showed significant reductions at 1 and 3 days for the HbV/rHSA group, at 3 and 7 days for the rHSA group, and at 3 days for the sRBC/rHSA group, but they returned to the original level at 14 days (Fig. 3).

Histopathological study

Histopathological examination revealed no significant changes in the lung, heart, and kidney in all groups. At 1 and 3 days after infusion, significant amounts of HbV phagocytized by macrophages in the marrow and Kupffer

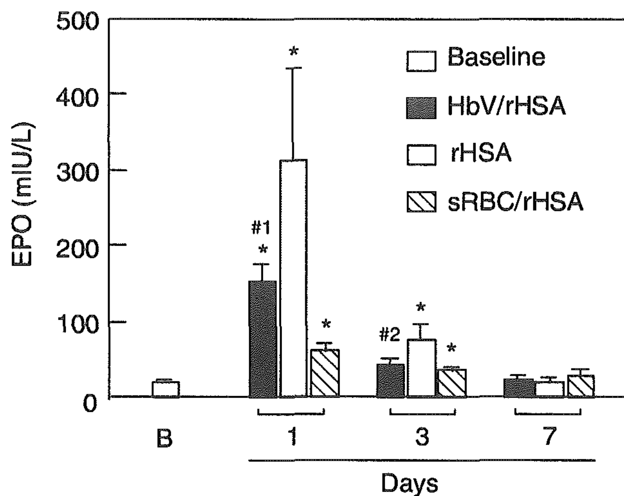

Fig. 2. Plasma EPO activity after 40 percent exchange transfusion with HbV/rHSA, rHSA, or sRBC/rHSA. All groups showed significant increases at 1 day. However, the HbV/rHSA groups showed a lower level than the rHSA group. The values are means ± SD. *Significantly different from the saline group ($p < 0.01$); #1p = 0.0222 versus rHSA; #2p = 0.0195 versus rHSA. B = baseline.

cells in the liver were observed. However, HbV decreased significantly at 7 days and was undetectable at 14 days. At 3 days after infusion, the pancreas in the HbV/rHSA group showed no significant morphological changes in spite of the increased lipase activity.

Sections of the spleen of the HbV/rHSA group, which is stained with Giemsa method, revealed the accumulation of HbV particles in the red pulp zone at 1 and 3 days after the exchange transfusion. The amount of the accumulated HbV decreased at 7 days and then became undetectable at 14 days (Fig. 4). Throughout the period examined in this study, nests composed of erythroblasts and proerythroblasts were formed in the splenic cord, especially at 3 and 7 days, indicating extramedullar erythropoiesis. Nest formation was remarkable for the rHSA group at 3 days. Hematopoietic activity was also observed at 3 days in the marrow of the HbV/rHSA group that contained erythroblastic islets.

The Berlin blue method indicated the presence of hemosiderin in macrophages of the spleen in the HbV/rHSA group at 7 days. This hemosiderin deposition increased until 14 days (Fig. 5). A small amount of hemosiderin was confirmed in the Kupffer cells of the liver at 14 days. Hemosiderin deposition, however, was undetected in the marrow. In addition, in the sRBC/rHSA group, hemosiderin deposition was present in the spleen macrophages at 14 days.

DISCUSSION

A main finding of this study is that the reduced Hct level after the 40 percent exchange transfusion with HbV/rHSA returned to the original level after 7 days; furthermore, the accumulated HbVs in RES became undetectable within 14 days. Significant splenomegaly is attributable to the combination of the accumulation of HbV in the red pulp zone and the considerable presence of nests of erythroblasts in the splenic cord in response to the EPO secretion, but these observations subsided within 14 days.

Extensive studies of circulation kinetics and organ distribution of isotope-labeled HbV clarified that HbV accumulates preferentially in the RES.[11,16] One cause of the splenomegaly is the accumulation of HbV particles in the red pulp zone, as shown in Fig. 4 but this subsided completely within 14 days. Gradual increases in the plasma cholesterol levels by 3 days after infusion and lack of disruption of the HbV in the plasma suggest that the cholesterols are liberated from the RES after the HbVs are captured by the RES and destroyed in the phagosomes of the macrophages.[14,15] In our previous studies of topload HbV infusions, significant increases in the high-density lipoprotein cholesterol, β-lipoprotein, and phospholipids were observed as surplus amounts.[15,17] In contrast, we observed no such significant increases after the 40 percent blood exchange, only decreases. A large demand of nutrients should pertain for hematopoiesis and so on; also, the lipid components from HbV might be utilized efficiently for proliferation.

During the metabolism of Hb, we would expect a release of bilirubin and iron. But they did not increase in the plasma within 14 days. The released heme from Hb in HbV might be metabolized by the inducible form of heme oxygenase-1 in the Kupffer cells of the liver and the spleen macrophages.[15,32] Bilirubin would normally be excreted in the bile as a normal pathway, and no obstruction or stasis of the bile should occur in the biliary tree. Berlin blue staining revealed considerable deposition of hemosiderin in the liver and spleen, even after 14 days. Normally, iron from a heme is stored in the ferritin molecule.[33] Both ferritin and hemosiderin release iron. They are anticipated to induce hydroxyl radical production followed by lipid peroxidation.[34] The iron release rate from hemosiderin, however, is substantially less than that from ferritin.[35] Consequently, the excess amount of iron would then normally be stored in an insoluble and less toxic form as hemosiderin. Hemosiderosis is often observed in patients who have received repeated blood transfusions because of the shorter $t_{1/2}$ of the stored RBCs. Moderate splenomegaly and hemosiderin deposition were also confirmed in the spleen in the sRBS/rHSA groups of this study, partly because of the accumulation and degradation of stored RBCs with the lowered membrane deformability and shortened circulation $t_{1/2}$.[26] These results indicate that the metabolism of heme from HbV and the iron storage is within the physiological capacity that has been well characterized for the metabolism of senescent RBCs.[36]

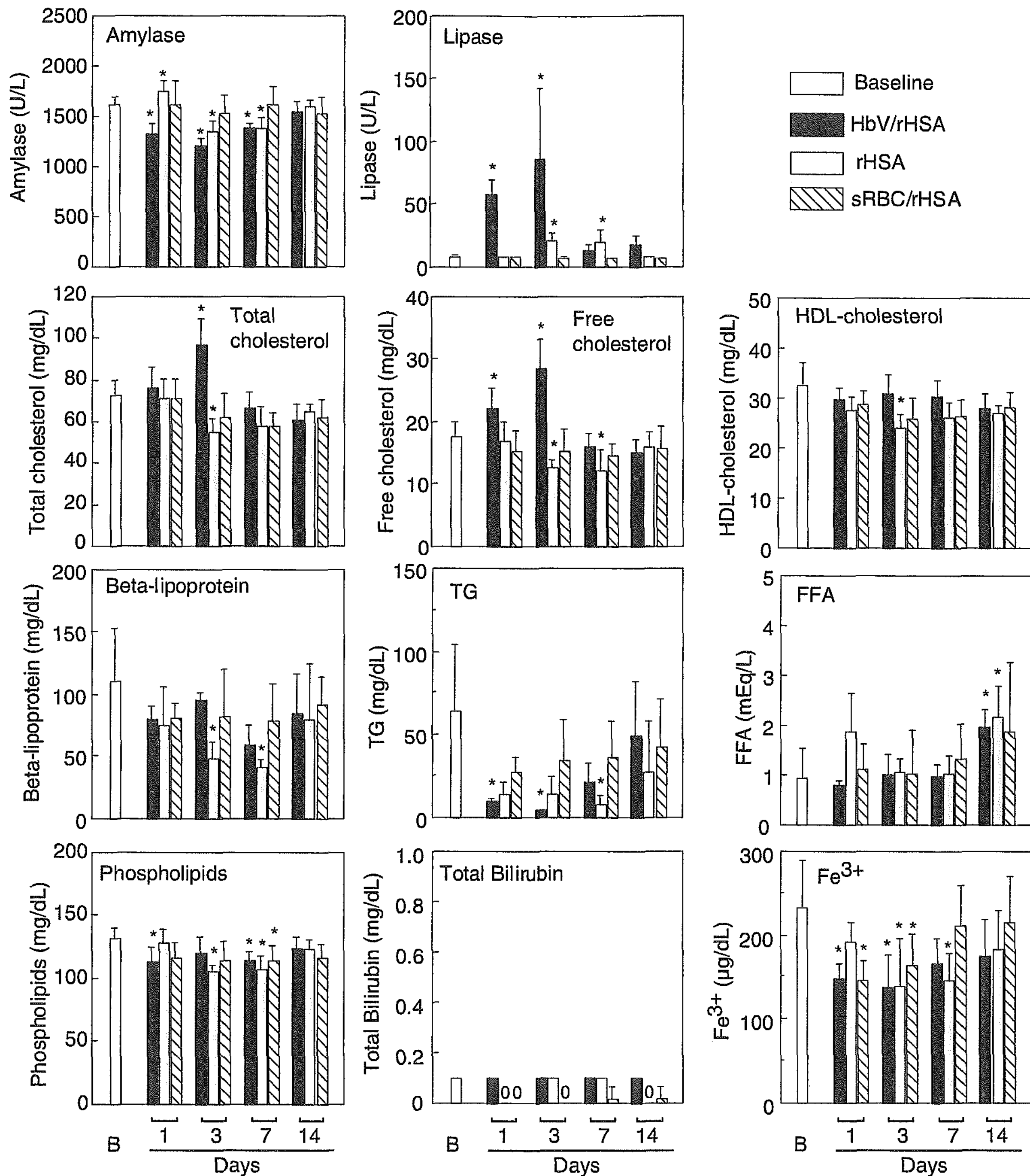

Fig. 3. Plasma biochemical tests representing the metabolism of the components of HbV (lipids and Hb) and pancreatic function after 40 percent exchange transfusion with HbV/rHSA, rHSA, or sRBC/rHSA. The values are means ± SD. *Significantly different from the saline group ($p < 0.01$). TG = triglyceride; FFA = free fatty acid; B = baseline.

Interestingly, not only the HbV/rHSA and sRBC/rHSA groups, but also the rHSA group showed a significant splenomegaly at 3 days, even though the rHSA group showed no symptoms on Day 1. In rats, extramedullary hematopoiesis induced by hypoxia is localized predominantly in the spleen.[37,38] We observed extensive nests of erythroblasts in the splenic cords, especially at 3 days. It is not plausible that the rHSA as an xenogeneic protein accumulates in the spleen macrophages, according to the fact the ^{125}I-labeled rHSA in a rat showed no specific distribution to the spleen.[39,40] Therefore, the splenomegaly for the rHSA group is attributed to the erythropoiesis stimulated by the significant increase in the plasma EPO level.

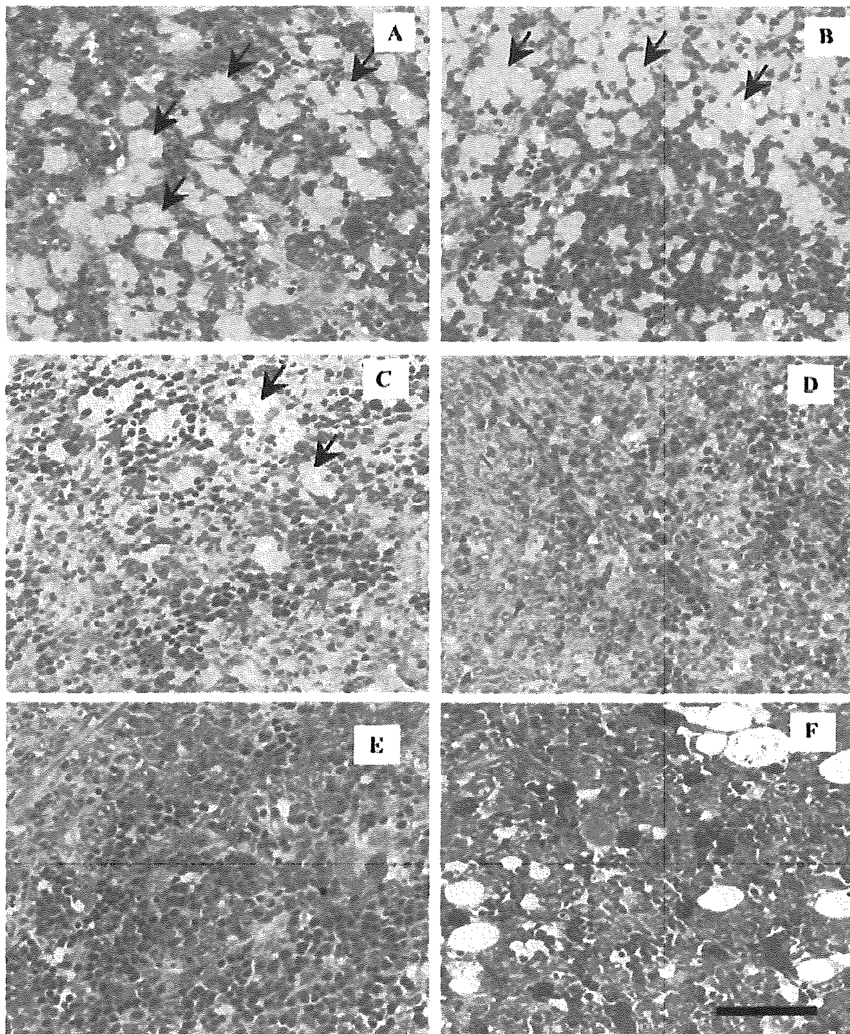

Fig. 4. Histology of rat spleen and marrow after exchange transfusion with HbV/rHSA or rHSA alone. (A-C) Respective images of the spleen of the HbV/rHSA group at 1, 3, and 7 days. Accumulated HbV particles are visible as light-blue areas (black arrows). Nests of erythroblasts are visible as dark blue cells (red arrows). The domain of the HbV particles decreased significantly at 7 days. (D) Spleen of the HbV/rHSA group at 14 days. HbV particles had disappeared, whereas the erythroblast nests remained, as indicated by the red arrows. (E) Spleen of the rHSA group at 3 days. The erythroblast nest formation is remarkable. (F) Marrow of the HbV/rHSA group at 3 days. Hematopoietic activity is visible. Bar = 50 μm (Giemsa method).

Plasma EPO release from the kidney strongly reflects an anemic condition, depending on the O_2-carrying capacity of the circulating blood.[41,42] The highest EPO level was seen in the rHSA group, indicating that its anemic condition was the most severe. Because of the short $t_{1/2}$ and MetHb formation,[43] the HbV/rHSA also showed a significant increase in the EPO level. However, it was considerably lower than that of the rHSA group. The sRBC/rHSA group also showed a moderate increase in the EPO level probably caused by the reduced Hct by the exchange transfusion. Accordingly, the splenomegaly for the HbV/rHSA and sRBC/rHSA groups is also partly attributable to the nests of erythroblasts for erythropoiesis that was sufficient for recovery from the reduced Hct. Interestingly, both HbV/rHSA and rHSA groups tended to show higher Hct values than the sRBC/rHSA group at 7 and 14 days, probably because of the enhanced erythropoiesis caused by the higher levels of EPO excretions than for the sRBC/rHSA group. The MCH, MCV, and MCHC levels were normal overall, supporting our inference of normal erythropoiesis.

Routine plasma biochemical tests showed that the hepatic function was maintained despite the large amount of HbV that were captured and degraded by Kupffer cells. Significant reductions were seen in the amylase activity, whereas a transient increase in lipase activity was observed consistently in our previous topload infusion experiments; this should be due to the up regulation of lipase in response to the infusion of phospholipid vesicles.[15,17,44]

In conclusion, all rats tolerated the 40 percent exchange transfusion with HbV/rHSA and showed complete Hct recovery within 7 days. Although transient splenomegaly and the hemosiderin deposition were confirmed, no excess iron was found in the blood. The recycling or excretion of iron as well as lipid components should be on the physiological pathway that is known for the degradation of senescent RBCs. Although some aspects remain unresolved, the present results offer important information on the safety and handling of HbV during preoperational or perioperational infusion in a clinical setting.

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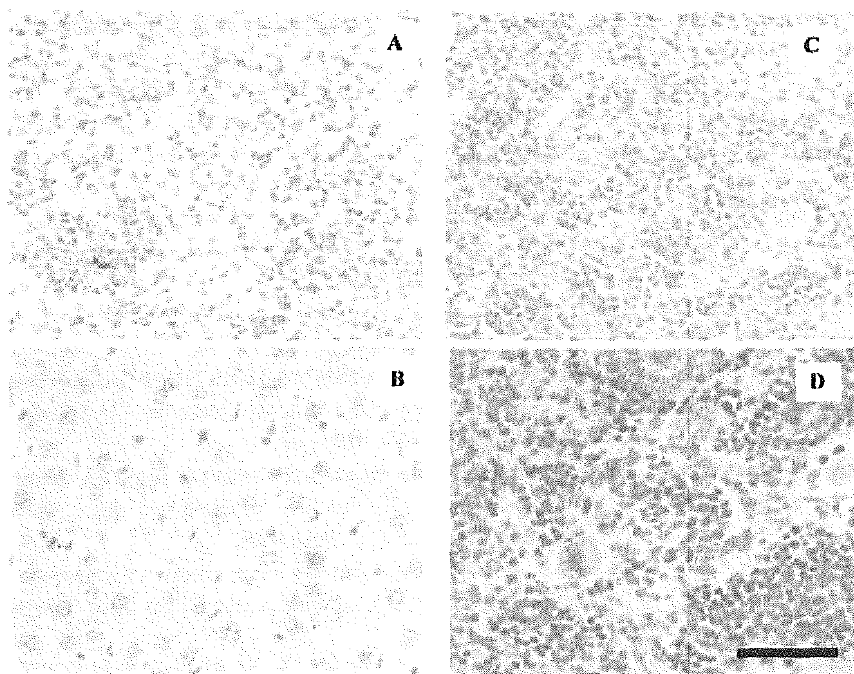

Fig. 5. Histology of rat spleen, liver, and marrow 14 days after exchange transfusion with HbV/rHSA or sRBC/rHSA. Spleen (A), liver (B), and marrow (C) of the HbV/rHSA group. The spleen and liver contained slight hemosiderin deposition, but not the marrow. The spleen of the sRBC/rHSA group (D) also contained slight hemosiderin deposition. Bar = 50 μm (Berlin blue method).

ful discussion of phagocytic and hematopoietic activities. The rHSA was obtained from Nipro Corp.

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The sealing effect of fibrin glue against alveolar air leakage evaluated up to 48 h; comparison between different methods of application

Masafumi Kawamura[a,*], Masatoshi Gika[a], Yotaro Izumi[a], Hirohisa Horinouchi[a], Noriko Shinya[b], Makio Mukai[c], Koichi Kobayashi[a]

[a]Division of General Thoracic Surgery, Department of Surgery, Keio University, School of Medicine, 35 Shinanomachi, Shinjuku-Ku, Tokyo, 160-8582 Japan
[b]Pathology Department, The Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan
[c]Division of Clinicopathology, Keio University Hospital, Tokyo, Japan

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Abstract

Objective: There is little experimental evidence to show how much positive airway pressure fibrin sealants can actually withstand, and in particular, how this effect changes over time. In the present study, we experimentally evaluated the sealing effect of fibrin glue against alveolar air leakage up to 48 h after application. **Methods:** Beagles were used (n=48). Under thoracotomy, approximately 5×10 mm defects (2 mm depth) were made on the lung surface. Fibrin glue sealants were applied to this defect in three ways. In rubbing and spray method, fibrinogen was rubbed, followed by spraying of both fibrinogen and thrombin solutions. In double layer method, fibrinogen was dripped, followed by thrombin. Collagen fleece, coated with fibrinogen and thrombin (TachoComb) was also tested. The minimum positive airway pressure which produced air leakage was measured for each sealed defect (seal breaking pressure, cmH_2O) at 0, 3, 6, 12, 24, and 48 h after application (n=6 at each time point). **Results:** The seal-breaking pressure increased over time in all of the application methods. At 6 h, differences between methods were not significant but three defects in RS reached 70 cmH_2O, the maximum pressure tested, compared with none in other two methods. At 12 h, the seal-breaking pressure was significantly higher in RS compared with the other two methods (rubbing and spray method vs TachoComb; 66 ± 3 vs 47 ± 17, P=0.047, rubbing and spray method vs double layer method; 66 ± 3 vs 42 ± 18, P=0.024). Beyond 24 h, sealing pressure reached close to 70 cmH_2O in all the methods. **Conclusions:** The results show that the sealing effect of fibrin glue is relatively unstable up to 12 h after its application. Rubbing and spray method may help the fibrin seal to reach its full strength faster compared with the other two methods.

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Keywords: Fibrin glue; Air leakage; Pulmonary resection; Sealing effect

1. Introduction

Alveolar air leakage is a very common complication in lung surgery. Along with inadequate control of postoperative pain, persistence of air leakage was identified as the most common cause of delay in hospital discharge after thoracic surgery [1]. Many tissue sealants are being applied to prevent air leakage after surgery [2-8]. Among them, fibrin glue is a popular sealant with a variety of application methods [9,10]. However, there are also reports that indicate that the use of fibrin glue does not reduce the duration of chest-tube drainage or hospital stay [11-13]. This implies that, air leakage often restarts shortly after surgery despite the application of fibrin glue.

Intraoperatively, we test the efficacy of fibrin glue by applying positive airway pressure. But we usually do not apply pressure beyond 20-25 cmH_2O, since it defeats the purpose to break the seal at this point. Clinically, it is not rare that air leakage becomes apparent, for example through the chest tube, shortly after surgery. While this may be air leakage from lesions that were missed during surgery, it is also true that airway pressure often spikes beyond the pressure tested, 25 cmH_2O, as the patient recovers spontaneous breathing. The fibrin seal may be broken at this point. To our knowledge, there is little experimental evidence to show how much positive airway pressure fibrin sealants can actually withstand, and in particular, how this effect changes over time. In the present study, we experimentally evaluated the sealing effect of fibrin glue against alveolar air leakage up to 48 h after application. We also compared three different methods of application.

2. Materials and methods

2.1. Animals

Adult male beagles, 10-12 months of age, weighing 8-11 kg were used for this study (Toyota Trading Co., Kumamoto, Japan) (n = 36). Animals were housed individually and provided

* Corresponding author. Tel.: +81 3 5363 3806; fax: +81 3 5363 3499.
E-mail address: kawamura@sc.itc.keio.ac.jp (M. Kawamura).

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food and water ad libitum. All animal studies were approved by the School of Medicine, Keio University Institutional Animal Care and Use Committee. All animals received humane care in accordance with the Japanese Government Animal Protection and management law

2.2. Fibrin glue application

The fibrin glue used in this study was Bolheal (The Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan). We also compared, fibrinogen-based collagen fleece, TachoComb (ZLB Behring Co., USA). The mechanism of fibrin glue formation is well described [14]. The fibrin glue product consists of two components. Solution A is a protein concentrate consisting of fibrinogen, plasma fibronectin, factor XIII, and plasminogen, reconstituted in aprotinin solution. Solution B is thrombin reconstituted in calcium chloride solution. TachoComb is a collagen fleece coated with dry fibrinogen and thrombin on one side.

We applied fibrin glue in two different ways, rubbing and spray method and double layer method. In rubbing and spray method, solution A was dripped and gently rubbed onto the air leakage area. Then both solutions were sprayed simultaneously onto the rubbed surface as a mixed aerosol using Bolheal Spray Set (The Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan). In double-layer method, solution A was dripped onto the air leakage surface after which solution B was dripped. To apply TachoComb, the fibrinogen-coated side was first soaked in saline, and then was attached to the air leakage surface. The sheet was gently pressed with dry gauze for about 5 min so that the collagen fleece was attached to the lung surface with fibrin glue.

2.3. Experiment

The dogs were anesthetized with an intravenous injection of pentobarbital sodium (25-30 mg/kg). The dogs were placed in left decubitus position, intubated, and mechanically ventilated. The right chest wall was shaved, and disinfected. Through a thoracotomy, defects were created on the right lung surface using scalpels, one on each of the three lobes (anterior, middle, and posterior). The defect was created with the lung fully inflated at a positive airway pressure of approximately 20 cmH_2O. The defect size was adjusted to be approximately 5×10 mm, and approximately 2 mm in depth. Hemostasis was obtained when necessary with the minimum use of electrocautery. In each animal, each of the three defects was sealed with one of three methods, rubbing and spray method, double-layer method, or TachoComb. Randomization was performed to allot these three methods to each lobe equally. The chest was closed, and the animals were allowed to recover, except for time 0. Xylazine (2 mg/kg) was administered as needed as analgesics. The minimum positive airway pressure which produced air leakage was measured for each sealed defect (seal-breaking pressure) at 0, 3, 6, 12, 24, and 48 h after the application of the fibrin sealant ($n=6$ at each time point). Except for time 0, thoracotomy was performed again under anesthesia. Air leakage pressure for each defect was evaluated separately by clamping the remaining two lobar bronchi with forceps. The maximum positive airway pressure applied was 70 cmH_2O, since higher pressure induced air leakage from uninjured lung around the hilum. After the completion of seal-breaking pressure measurement at each time point, each animal was sacrificed with intracardiac injections of pentobarbital (1000 mg/body).

2.4. Histological examinations

A separate group of animals was used to obtain histological specimens because the fibrin seal is broken by the seal breaking pressure measurements. Two specimens for each method and time points were prepared ($n=12$). The animals were sacrificed and the whole right lung was fixed in 10% neutral formaline. After fixation, each defect site was resected, embedded in paraffin, and processed in 3 μm sections for hematoxylin-eosin staining. Specimens were analyzed at Clinicopathological Division of Keio University Hospital in a blinded manner by M.M.

2.5. Statistical analyses

The results are presented as the mean $\pm$ SD. Seal-breaking pressure per time point was compared between different methods using unpaired T-test. Differences within each method were tested using paired T-test. Significance was assumed at $P<0.05$.

3. Results

3.1. Seal breaking pressure measurements

The seal-breaking pressure increased over time in all the application methods (Fig. 1). At 0 h, seal-breaking pressure was significantly higher in rubbing and spray method compared with TachoComb (54 ± 5 vs 36 ± 6, $P<0.001$), and in TachoComb significantly higher compared with double layer method (36 ± 6 vs 27 ± 3, $P=0.007$). Seal breaking

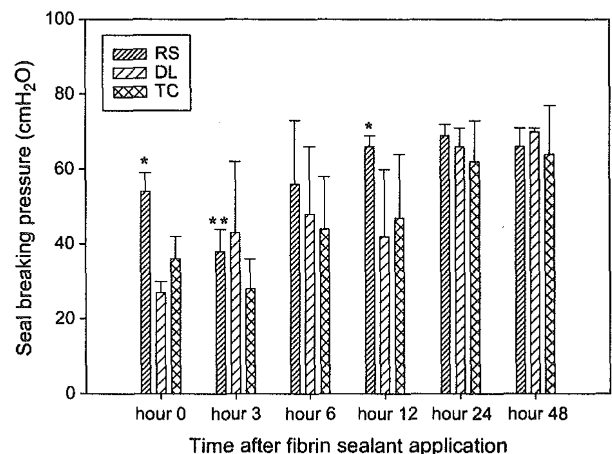

Fig. 1. The time interval changes in seal-breaking pressure after application of the fibrin sealants is shown. RS, rubbing and spraying method; DL, double layer method, TC, TacoComb. *$P<0.05$ vs other two groups, **$P<0.05$ vs RS at 0 h.

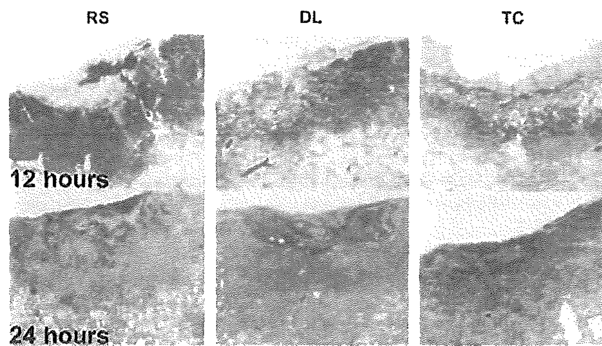

Fig. 2. Hematoxylin-eosin staining of the injured lung sealed by different fibrin sealants. At 12 h, deeper penetration of fibrin into the injured lung parenchyma was seen in RS compared with the other two methods. This difference was not apparent between application methods beyond 24 h. Upper and lower panels correspond, respectively, to 12, and 24 h after sealant application RS, rubbing and spraying method; DL, double layer method; TC, TacoComb, magnification 2×.

pressure in rubbing and spray method declined significantly from 0 to 3 h (from 54±5 to 38±6, $P<0.001$). At 3 h, seal-breaking pressure in double layer method tended to increase, and in TachoComb tended to decline compared with 0 h, but these changes were not significant. At 6 h, differences between methods were not significant but three defects in rubbing and spray method reached 70 cmH_2O, the maximum pressure tested, compared with none in other two methods. At 12 h, the seal-breaking pressure was significantly higher in rubbing and spray method compared with the other two methods (rubbing and spray method vs TachoComb; 66±3 vs 47±17, $P=0.047$, rubbing and spray method vs double layer method; 66±3 vs 42±18, $P=0.024$). Beyond 24 h, sealing pressure reached close to 70 cmH_2O in all the methods, with no significant differences between methods.

3.2. *Histological examinations*

The layer of fibrin covering the lung surface could be observed in all three methods by hematoxylin-eosin staining (Fig. 2). Up to 12 h, deeper penetration of fibrin into the injured lung parenchyma was seen in rubbing and spray method compared with the other two methods. This difference was not apparent between application methods beyond 24 h. Also, at 3 h, hemorrhage was more evident underneath the fibrin layer in rubbing and spray method compared with other two methods.

4. Discussion

Fibrin glue is derived from human, or in some products like bovine plasma, and hence, carry the same risks as blood transfusion. We have reported the possibility of viral transmission by clinical use of fibrin glue [16]. Despite these potential drawbacks, fibrin glue is widely used in order to reduce postoperative alveolar air leakage, but questions remain regarding its clinical efficacy [5,12,13,15]. The results of this study show that the sealing effect of fibrin glue is relatively unstable up to 12 h after its application. Clinically, this result suggests that coughing or positive pressure ventilation should be kept to a minimum for 12 h in order to fully exploit the sealing effect of fibrin glue.

The sealing effect of fibrin glue is affected by the concentration of fibrin, and how well it attaches to tissue. The concentration of fibrin depends primarily on how well the thrombin and fibrinogen solutions are mixed on application. The attachment of fibrin may be affected at least in part by its penetration into tissue. Rubbing and spray method is a method that we have recently devised. Our intention was to improve tissue penetration by rubbing fibrinogen into the lung parenchyma. We also utilized the effective mixing of the two solutions by aerosol to form a more even layer of fibrin in continuity with the penetrated fibrinogen, which is converted to fibrin by the spray. The present study suggests that rubbing and spray method may help the fibrin seal to reach its full strength faster compared with the other two methods. Histological findings, at least in part suggest that this may be due to the initial deeper penetration of fibrin into the lung parenchyma. We speculate that because of this, the attached surface area of fibrin was initially greater in rubbing and spray method compared with the other two methods. Presumably, this difference became insignificant with the formation of tissue-derived fibrin. We evaluated TachoComb and double layer method as the most widely used methods. Double layer method is the application method recommended by most manufacturers, and is therefore, presumably most often used. It is encouraging that both these methods provided satisfactory sealing effect beyond 24 h. Control experiments, in which no sealant was used, was not performed due to ethical reasons. In our preliminary studies, the alveolar leakage created in this experiment did not stop spontaneously, and respiratory distress was unavoidable even with the use of chest tubes. Regarding rubbing and spray method, there was haemorrhage underneath the fibrin layer at 3 h, which resolved at 6 h. Presumably this was caused by rubbing. This may in part explain the significant decrease in seal-breaking pressure in rubbing and spray method at 3 h. A less invasive way to infiltrate the fibrinogen solution, for instance the use of a soft sponge, is currently being studied.

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Role of Rho-kinase in reexpansion pulmonary edema in rabbits

Makoto Sawafuji,[1] Akitoshi Ishizaka,[2] Mitsutomo Kohno,[1] Hidefumi Koh,[2] Sadatomo Tasaka,[2] Yoshiki Ishii,[3] and Koichi Kobayashi[1]

Departments of [1]Surgery and [2]Medicine, Keio University School of Medicine, Tokyo; and [3]Department of Pulmonary Medicine and Clinical Immunology, Dokkyo University School of Medicine, Tochigi, Japan

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Sawafuji, Makoto, Akitoshi Ishizaka, Mitsutomo Kohno, Hidefumi Koh, Sadatomo Tasaka, Yoshiki Ishii, and Koichi Kobayashi. Role of Rho-kinase in reexpansion pulmonary edema in rabbits. *Am J Physiol Lung Cell Mol Physiol* 289: L946–L953, 2005. First published July 8, 2005; doi:10.1152/ajplung.00188.2004.—Reexpansion of a collapsed lung increases the microvascular permeability and causes reexpansion pulmonary edema. Neutrophils and their products have been implicated in the development of this phenomenon. The small GTP-binding proteins Rho and its target Rho-kinase (ROCK) regulate endothelial permeability, although their roles in reexpansion pulmonary edema remain unclear. We studied the contribution of ROCK to pulmonary endothelial and epithelial permeability in a rabbit model of this disorder. Endothelial and epithelial permeability was assessed by measuring the tissue-to-plasma (T/P) and bronchoalveolar lavage (BAL) fluid-to-plasma (B/P) ratios with ^{125}I-labeled albumin. After intratracheal instillation of ^{125}I-albumin, epithelial permeability was also assessed from the plasma leak (PL) index, the ratio of ^{125}I-albumin in plasma/total amount of instilled ^{125}I-albumin. T/P, B/P, and PL index were significantly increased in the reexpanded lung. These increases were attenuated by pretreatment with Y-27632, a specific ROCK inhibitor. However, neutrophil influx, neutrophil elastase activity, and malondialdehyde concentrations in BAL fluid collected from the reexpanded lung were not changed by Y-27632. In endothelial monolayers, Y-27632 significantly attenuated the H_2O_2-induced increase in permeability and mitigated the morphological changes in the actin microfilament cytoskeleton of endothelial cells. These in vivo and in vitro observations suggest that the Rho/ROCK pathway contributes to the increase in alveolar barrier permeability associated with reexpansion pulmonary edema.

acute lung injury; acute respiratory distress syndrome; reexpansion pulmonary edema; Rho; Rho-kinase

REEXPANSION OF A COLLAPSED lung occasionally causes acute lung injury (ALI) known as reexpansion pulmonary edema (3, 17, 18). The main characteristic of this disorder is an increase in pulmonary microvascular permeability (24, 40). Vascular endothelial permeability is related to the cellular cytoskeleton. An increase in vascular permeability is accompanied by a reorganization of the actin-based cytoskeleton and contraction of endothelial cells, resulting in the formation of an intercellular gap (7, 28). A calmodulin-dependent myosin light chain kinase (MLCK) contributes to the regulation of cell contraction and endothelial cell permeability. Activation of MLCK causes the phosphorylation of the myosin light chains, resulting in the contraction of actomyosin, in the formation of stress fibers and in subsequent hyperpermeability of endothelial cells (8). A signaling pathway of the small GTPase Rho and its target protein, Rho-kinase (Rho-associated coiled-coil-forming protein kinase; ROCK), also regulates the contraction of endothelial cells (5). The activation of Rho leads to the phosphorylation of the myosin binding subunit of myosin light chain phosphatase via the action of ROCK, inactivating the myosin light chain phosphatase and blocking the dephosphorylation of myosin light chains (16). Continued phosphorylation of myosin light chains results in sustained cell contraction.

In studies in isolated rat lungs, a MLCK inhibitor attenuated the injury associated with ischemia-reperfusion (15) and that induced by ventilators (23). These observations suggest that the intracellular signal transduction that regulates the cellular cytoskeleton is involved in the pathophysiology of some forms of ALI. However, the role of the Rho/ROCK-mediated pathway in reexpansion pulmonary edema in vivo remains unclear, although more attention has been paid to the role of alveolar epithelial injury in the pathophysiology of ALI (38). The Rho/ROCK-mediated pathway has been implicated in the organization of perijunctional actin and in the regulation of permeability of the tight junction of the epithelial cells (6, 22). Therefore, we hypothesized that the Rho/ROCK-mediated pathway is involved in the changes in alveolar endothelial and epithelial barrier permeability in reexpansion pulmonary edema.

Y-27632, a highly selective inhibitor of ROCK in vitro and in vivo (36), was found useful to examine the role of the Rho/ROCK-mediated pathway in vivo (11, 29). To test our hypothesis, we examined the inhibitory effect of Y-27632 in our rabbit model of reexpansion lung injury (20). We measured the flux of transvascular albumin as an indicator of endothelial permeability (10). Alveolar epithelial permeability was assessed by measuring the bidirectional flux of albumin across the alveolar epithelial barrier (10, 30, 39). We also performed in vitro experiments in human pulmonary artery endothelial cell monolayers to study the effects of Y-27632 on hyperpermeability and endothelial cytoskeletal rearrangement induced by H_2O_2. These experiments were performed since reactive oxygen species appear to be involved in the injury to pulmonary endothelial cells observed in reexpansion pulmonary edema (12). We observed morphological changes in the microfilament cytoskeleton using a rhodamine-phalloidin stain (25). The main objective of this study was to ascertain the role of the Rho/ROCK pathway in the increased alveolar endothelial and epithelial barrier permeability present in reexpanded lungs.

METHODS

The experiments were performed in 45 male Japanese White rabbits, weighing between 900 and 1,800 g. All procedures were

Address for reprint requests and other correspondence: M. Sawafuji, Dept. of Surgery, Keio Univ., 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan (e-mail: msawafuji@nifty.com).

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reviewed and approved by the Laboratory Animal Care Panel of Keio University. The animals were divided into the following three groups: *1*) a reexpansion group (RE), *2*) a Rho-kinase inhibitor pretreatment group (RE + Y), which received 10 mg/kg Y-27632 intraperitoneally before reexpansion of the left lung, and *3*) a sham-operated group (sham) that underwent thoracotomy only. The surgical techniques have been previously reported in detail (20). Briefly, general anesthesia was induced with intramuscular ketamine (75 mg/kg) and xylazine (5 mg/kg). A tracheostomy was performed, and a 3.5-mm-diameter Portex LTD tracheal cannula (Hythe, Kent, UK) was inserted. The rabbits were mechanically ventilated (SN-480–7; Shinano, Tokyo, Japan) with room air at 30 breaths/min and a tidal volume of 10 ml/kg.

In the RE and RE + Y groups, after completion of the left posterolateral thoracotomy, the left main bronchus was clamped with an atraumatic vascular clip (Vascu-statt; Scanlan International, St. Paul, MN) to produce complete collapse of the left lung by absorption of the alveolar gas in the pulmonary circulation. A 4–0 nylon string attached to the clip was exteriorized for later retraction. In the sham group, a left posterolateral thoracotomy was performed, and the left main bronchus was dissected from the surrounding tissue. The chest wall was closed. The rabbits were allowed to recover for 36 h before the experiments began. The tracheal cannula was removed after the recovery of spontaneous breathing.

Experimental Protocol

Experiment 1. Twenty-one animals were divided into three groups listed above to measure endothelial and epithelial permeability. The protocol for *experiment 1* is provided in Fig. 1. Endothelial permeability was assessed from the transvascular flux of ^{125}I-labeled albumin administered intravenously. Epithelial permeability was measured as the flux of ^{125}I-albumin from intravascular space to airspace. To correct blood contamination in the lung tissue or bronchoalveolar lavage (BAL) fluid samples, ^{131}I-albumin was injected intravenously. On the day of experiments, the rabbits were sedated with intramuscular ketamine (100 mg/kg) 30 min before the experiment and placed in the supine position, and a 24-gauge catheter was inserted in an ear vein. ^{125}I-labeled BSA (2 ml of 37 kBq/ml; Life Science Products, Boston, MA) was injected intravenously to assess the pulmonary extravasated albumin in all experimental groups. In the RE and RE + Y groups, 10 min after ^{125}I-albumin injection, the left main bronchus was reopened by retracting the string. At this time, a 9-Fr drainage chest tube (Sumitomo Bakelite, Tokyo, Japan) was inserted in the left pleural cavity, and continuous suction at −10 cmH$_2$O was applied through a chest tube to reinflate the collapsed lung. In the RE + Y group, 10 mg/kg Y-27632 were administered in the peritoneal cavity 30 min before reopening the left main bronchus. An identical volume of saline was injected intraperitoneally in the sham and RE groups. Room air breathing resumed spontaneously. To prevent hypotension and dehydration, 50 ml/kg normal saline were infused intravenously throughout the period of continuous suction. ^{131}I-labeled human serum albumin (2 ml of 37 kBq/ml; Daiichi Radioisotope Laboratories, Tokyo, Japan) was injected intravenously 115 min after reopening the left main bronchus. We used ^{131}I-albumin as a plasma volume marker to correct for residual blood content in excised lung tissue samples. Heparin (2,000 IU iv) was injected simultaneously. The animals were killed 120 min after reopening the left main bronchus by injection of 100 mg pentobarbital intravenously. The chest was immediately opened, 5 ml blood were collected by cardiac puncture, each pulmonary hilus was clamped at the end of expiration, and the lungs were removed and divided into superior and inferior lobes. Blood and superior lobe lung samples were placed in a preweighed glass tube for measurements of weight and radioactivity.

Total blood cells were counted with a Sysmex K-1000 counter adapted for rabbit cells (Sysmex, Kobe, Japan). Differential counts of 200 cells were performed on glass slide smears stained by a modified Wright's stain (Diff-Quick; American Scientific, McGraw Park, IL).

BAL was performed with 10 ml normal saline through a catheter secured within the bronchus of the excised inferior lobe. The volume of fluid recovered from the left lung of the sham, RE, and RE + Y groups were 7.9 ± 0.3, 8.1 ± 0.4, and 8.0 ± 0.5 ml, respectively. The corresponding volumes recovered from the right lung were 7.9 ± 0.4, 8.3 ± 0.3, and 8.3 ± 0.5 ml, respectively. There was no significant difference in the recovery of BAL fluid among the groups. The fluid was centrifuged at 400 *g* and 4°C for 10 min. The supernatant was used for gamma counting of ^{125}I and ^{131}I to measure the pulmonary epithelial permeability, and for the analysis of neutrophil elastase activity and malondialdehyde (MDA) concentration. The cell pellet was resuspended in 1 ml saline, and the cells were counted by a modified hemocytometer method (Unopet Microcollection System; Becton-Dickson, Rutherford, NJ). Cell differential in BAL was counted in smears obtained by cytocentrifugation. Differential counts were made on 200 cells from smears stained with a modified Wright's stain.

In nine additional animals, divided into the three experimental groups and treated similarly, the mean arterial pressure was measured with a polygraph (Nihon Koden, Tokyo, Japan) via a catheter inserted in the carotid artery.

Experiment 2. Twelve animals were divided among the three experimental groups described earlier to measure the epithelial permeability. Epithelial permeability was measured as the flux from the airspace to intravascular space of ^{125}I-albumin instilled in the left lung. On the day of experiments, 5 ml blood were collected from a catheter inserted in the right carotid artery, heparinized, and centrifuged to obtain plasma. The blood was replaced with an equal volume of Ringer lactate. After the onset of continuous suction (5 min), the rabbits were placed in the left lateral decubitus position to facilitate the collection of liquid in the left lung. Autologous plasma (2 ml/kg) containing 3 mg Evan's blue dye and 212 kBq ^{125}I-labeled BSA were instilled in the left inferior lobe over 5 min with an 18-gauge tube advanced through the tracheostomy, as previously described (30, 39)

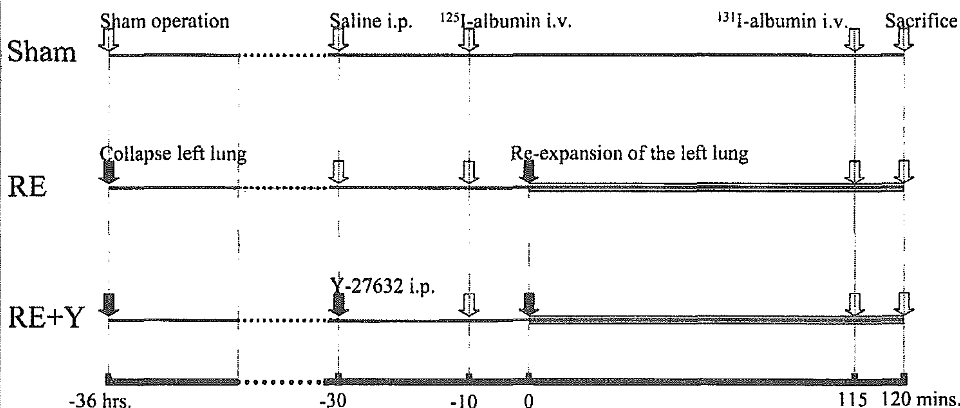

Fig. 1. Protocol for *experiment 1*. The experimental groups consist of the following: sham-operated group (Sham), reexpansion group (RE), and Rho-kinase inhibitor pretreatment group (RE + Y). Sham group, only thoracotomy was performed. RE group, left main bronchus was declamped after 36 h of clamping, and the collapsed lung was reexpanded for 2 h. RE + Y group, 10 mg/kg specific Rho-kinase inhibitor (Y-27632) was administered in the peritoneal cavity 30 min before reopening the left main bronchus. ^{125}I-albumin was injected iv 10 min before reopening the left main bronchus to assess transvascular permeability. ^{131}I-albumin was injected iv 5 min before death to correct blood contamination.